INVESTOR IN PEOPLE

BEQ3/00114

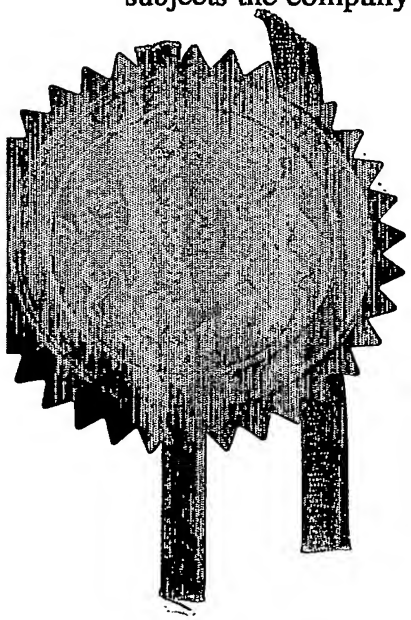
The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Signed Stephen Hordley

Dated 15 July 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference LRD-GB-1-418

2. Patent application number *(The Patent Office will fill in this part)* 0214749.4 26 JUN 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

K.U.Leuven Research and Development - Groot Begijnhof 59 - 3000 Leuven

Represented by Dr. Ivo Roelants, IPR Officer

Patents ADP number *(if you know it)* 77909750 01 IT

If the applicant is a corporate body, give the country/state of its incorporation Belgium

4. Title of the invention

TIME TEMPERATURE INTEGRATORS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

K.U.Leuven R&D
care off:
Hubert Velge
Neaves Cottage
Neaves Lane - Glyndebourne
East Sussex BN8 5UA

Patents ADP number *(if you know it)* 80491650 02 I

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	57
Claim(s)	1
Abstract	1
Drawing(s)	16 only 8

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	1
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	1 fax cover sheet 1 fee sheet with request for fax back service

11. I/We request the grant of a patent on the basis of this application.

Dr. Ivo Roelants - IPR Officer — Signature — Date 26/02/2002

12. Name and daytime telephone number of person to contact in the United Kingdom: Hubert Velge +44 7940 540 397

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

TIME TEMPERATURE INTEGRATORS

FIELD OF THE INVENTION

Nowadays, an important part of our food supply consists of foods preserved through a thermal treatment such as blanching, pasteurisation, sterilisation, etc... In this context it has been and is still important for industrials, to measure the impact of thermal processes in term of safety and quality in the context of process design, optimization, evaluation and control. The in-situ method and the physical-mathematical approach are the two commonly used evaluation techniques: In the in-situ method the level of a quality/safety attribute (i.e. thiamine content) is measured in the food itself before and after heat treatment while the physical-mathematical method is based on the knowledge of the time-temperature history of the product, combined with the knowledge of heat resistance parameters of the food quality attribute under study.

Increasing safety and quality requirements, energy saving policies and consumers aspects, have resulted in the optimization of classical thermal technologies and the development and application of new (thermal as well as non thermal) technologies such as continuous processing in rotary retorts, volumetric heating before aseptic processing, use of high electric field pulses , high pressure processing, Infra-Red technology etc... Confronted with these new technologies, the in-situ and physical-mathematical methods show serious limitations. This is why considerable efforts have been and will continue to be put into the development of product history integrators such as Time Temperature Integrators (TTIs) for thermal processing. TTIs allow to determine post factum fast, easily and accurately the impact of a thermal treatment on a product attribute without knowledge of the time-temperature history of the product.

The present paper focuses on the development of TTIs to monitor thermal processes from a safety point of view. Until now, mainly microbiological TTIs are used for this purpose. However, the inherent disadvantages associated with microbiological detection methods have encouraged the investigation of alternatives. The study of the advantages and drawbacks of several kinds of TTIs (Hendrickx *et al.*, 1993, 1995, Van Loey *et al.*, 1996), encouraged these authors to focus on the study of TTIs based on thermostable enzymes.

Several enzymic systems have been developed to evaluate the impact of pasteurization processes (Van Loey *et al.*, 1995a-b, 1996, De Cordt, 1992) and sterilization processes (Van Loey *et al*, 1997a-b, Haentjens et al., 1998) on, among others, microbiological target attributes such as *Clostridium botulinum* spores. Although these α-amylase based TTI systems are valuable research tools, the need of an enzymic system that is accurate, inexpensive and convenient to use for the monitoring of industrial sterilization processes is still existing at this moment.

The present paper, after a detailed introduction on quantitative evaluation of thermal process impact and on the TTI concept, presents an original standardized preparation procedure, in terms of materials, methods and results, allowing to prepare enzymic TTIs for the monitoring of thermal process impacts from a safety point of view.

1.CONCEPT AND METHODS FOR QUANTITATIVE EVALUATION OF THERMAL PROCESSES

1.1 Introduction

The aim of thermal processing is to preserve food against spoilage which could render the food unfit for consumption, by inactivation of pathogenic and/or spoilage microorganisms and of enzymes with deteriorative action. Unfortunately, along with these desired effects, thermal processing also affects both the nutritional and sensorial quality of the product. It is the challenge of the food technologist to optimize the processes, namely to minimize quality losses and at the same time providing an adequate process to achieve the desired degree of lethality. This optimization is possible because of the more pronounced temperature dependence of microbial (spores) inactivation as compared to the rate of quality destruction, sensorial as well as nutritional (Lund, 1977).

Hence, relevant knowledge of the kinetic data for heat induced inactivation of microorganisms and quality evolution is essential to optimize thermal processes (Van Loey *et al.*, 1994 a-c).

In addition, for preservation purposes, the quantitative measurement of the impact of a thermal process in terms of safety and quality is important for process design, evaluation and optimization to obtain a safe food product with maximal quality retention. In the present section, the concept and methods commonly used to quantify the impact of a thermal process are presented.

1.2 Quantification of the thermal impact on a product aspect

The impact of a heat treatment on a product safety or quality attribute depends on the rates of the heat-induced reactions that affect this attribute, and on the time interval during which these reactions occur.

The 'status' of a food, expressed in terms of its safety or quality, is determined by the effect of all reactions occurring in the product, integrated over the full history of the product until the moment of consumption. Figure 1.1 shows the idea of the « preservation reactor » (Van Loey *et al.*, 1996). This concept can be applied for an entire production chain : preparation and packaging, processing, distribution and storage or it is applicable to a single unit operation. The rates at which desired and undesired reactions (related to food safety and quality) take place are function of both intrinsic (i.e. food specific) properties and extrinsic (i.e. process specific) factors (Maesmans *et al.*, 1990, Van Loey *et al.*, 1996). In general, an n^{th} order reaction rate equation can be written as equation 1.1.

$$\frac{dX}{dt} = -k X^n \qquad (1.1)$$

Where X is the response value at time t (e.g. microbial count, nutrient concentration), k the reaction rate constant and n the order of the reaction.

Because in thermal processing of foods, temperature is the main extrinsic factor for guaranteeing safety and quality during the production and storage of food products, the following discussion is limited to systems for which temperature is the only rate determining extrinsic factor.

1.2.1 Kinetic models to describe the influence of temperature on the reaction rate constant

Two terminologies are commonly used to quantify the influence of temperature on the inactivation rate of safety as well as of quality aspects: the Arrhenius model (1889) commonly used in the field of chemical reaction kinetics and the Thermal Death Time model (TDT) (Bigelow, 1921), especially to describe first order kinetics (n=1 in equation (1.1)) and commonly used in thermobacteriology and in the field of thermal processing. These two models are described in box 1. The best known and used theory in the area of biological engineering and chemical reaction kinetics is that proposed by Arrhenius (1889), which is applicable to reactions in solutions and heterogeneous processes. According to Arrhenius, the temperature dependence of the rate constant k can be expressed as equation (1.2)

$$k = k_{ref} \cdot \exp\left(\frac{E_a}{R_g}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right) \qquad (1.2)$$

where Ea is the activation energy, k_{ref} is the rate constant at reference temperature (T_{ref}) and R_g is the universal gas constant.

In thermobacteriology, however, the TDT concept of Bigelow (1921) is commonly used to describe heat inactivation kinetics of first order reactions. The decimal reduction time, D-value (min), is defined as the time required, at a constant temperature, to reduce the initial response value (X0) by 90% or in other words, the time at a constant temperature to traverse one log cycle. Hence, for first order reactions the relation between the decimal reduction time D and the reaction rate constant k is given by equation (1.3)

$$D = \frac{\ln 10}{k} \qquad (1.3)$$

Bigelow (1921) reported that if the logarithms of the destruction times (D-values) are plotted versus temperature on a logarithmic scale, the result over the usual range of temperatures of interest can be represented by a straight line. Hence, the temperature dependence of the D-value is given by the z-value which is the temperature increase required to obtain a tenfold decrease of the D-value (equation 1.4)

$$D = D_{ref} \cdot 10^{\left(\frac{Tref - T}{z}\right)} \qquad (1.4)$$

where D_{ref} is the decimal reduction time at a reference temperature T_{ref}, T the actual temperature and z the z-value of the system.

1.2.2. Concept to express the integrated time-temperature impact

The number of decimal reductions inflicted on the target attribute(s) under study defines the impact of the thermal process.

The impact of a thermal treatment on a food attribute is indeed usually quantified using the concept of an « equivalent time at a reference temperature », referred to as the processing value F (usually expressed in minutes). This concept translates the time-temperature variable profile into an equivalent time chosen at a constant reference temperature that will affect the quality attribute in the same way as the variable profile. In other words, F represents the equivalent time at a chosen constant reference temperature T_{ref} that would result in exactly the same impact on the specific quality attribute as the actual time-temperature variable profile to which the food (i.e. the attribute of interest) was subjected (Van Loey, 1998).

Mathematically, the processing value can be written in terms of the temperature history of the product or alternatively in terms of a response status before and after processing. The impact of a heat treatment on a product safety or quality attribute depends on the rates of the heat-induced reactions affecting this attribute and on the reaction time interval during which these reactions rates occur.

In practice, isothermal heating profiles almost never occur because heating equipments require time to reach the process holding temperature (CUT: Come Up Time) and require time for cooling

(CDT: Come Down Time) and because of the heat transfer inside the product. Hence, reaction rates are varying as a function of process time. The process-value is defined as the integral over time of the rate at each encountered temperature relative to the rate at the chosen reference temperature T_{ref} denoted as subscript (eq.1.5).

$$E_a F_{T_{ref}} = \int_0^t \frac{k}{k_{ref}} \cdot dt \qquad (1.5)$$

Where F is the process value, k the rate constant at T and k_{ref} the rate constant at reference temperature T_{ref}.
An analogue expression for the process value as in equation (1.6) can be obtained by combination of equation (1.3) with equation (1.5):

$$^{z}F_{T_{ref}} = \int_0^t \frac{D_{ref}}{D} \cdot dt \qquad (1.6)$$

Where F is the process-value, D the decimal reduction time at T and D_{ref} the decimal reduction time at reference temperature T_{ref}.

Combination of equation (1.2) and (1.5) -in case of the Arrhenius terminology gives a first set of expressions (eq. 1.4 and 1.6) to determine the impact of a thermal process on a specific product aspect, characterized by its activation energy (z-value), denoted as superscript.

$$^{E_a}F_{T_{ref}} = \int_0^t \exp\left[\frac{E_a}{R_g}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \cdot dt \qquad (1.7)$$

$$^{z}F_{T_{ref}} = \int_0^t 10^{\left(\frac{T - T_{ref}}{z}\right)} \cdot dt \qquad (1.8)$$

Hence, based on the temperature history of the product aspect, either recorded or simulated, combined with knowledge on the kinetics of the monitored aspect, the thermal process impact on that attribute can be calculated as mentioned above, this approach is commonly referred to as the physical mathematical method (see (1.3)).

The general expression of a first order decay can be rewritten as equation (1.9) and of an nth order reaction (n≠1) as equation (1.10):

$$\log\left(\frac{X}{X_0}\right) = -\int_0^t \frac{dt}{D} = -\int_0^t \frac{k}{2.303}\,dt \qquad (1.9)$$

$$\frac{X^{1-n} - X_0^{1-n}}{1-n} = -\int_0^t k\,dt \qquad (1.10)$$

Hence, the impact of a thermal process on a specific parameter can also be determined relying solely on the initial and final status of the parameter of interest and on its kinetics. These expressions are obtained by combination of equation (1.6) with equation (1.9) and of equation (1.5) with equation (1.10) and are given below for a parameter that obeys a first order decay (eq. 1.11)) or a nth order (n≠1) decay (eq. 1.12).

$$^{z(E_a)}F_{T_{ref}} = D_{ref}\log\left(\frac{X_0}{X}\right) = \frac{1}{k_{ref}}\ln\left(\frac{X_0}{X}\right) \qquad (1.11)$$

$$^{E_a}F_{T_{ref}} = \frac{1}{k_{ref}}\left(\frac{X^{1-n} - X_0^{1-n}}{n-1}\right) \qquad (1.12)$$

Based on the response status of an attribute before (X_0) and after (X) thermal treatment, combined with its kinetics, the process impact can be calculated using equation (1.11) or (1.12) depending on the order of the heat induced reaction occurring to this attribute. In case the level of the actual quality attribute of interest is measured before and after processing, this approach is referred to as the in-situ method (see (1.3)).

1.3 Methods for process assessment: the need for TTIs.

As briefly mentioned above, two well-documented and well-elaborated techniques, which are most commonly used to evaluate thermal processes, are the in-situ approach and the physical-mathematical method.

In the in situ method, changes in the actual quality or safety attribute under study are monitored before and after processing to provide direct and accurate information on the status of the

attribute of interest. The processing value can be calculated using equation (1.11) or (1.12) depending on the order of the heat-induced reaction occurring to the target attribute. The main advantage of the in-situ method is that the impact of the process on the parameter of interest is directly and accurately known. However, in practice, the analysis of the parameter under investigation (i.e. measurement of microbial counts, texture, vitamin content, etc.), can be laborious, time-consuming and/or expensive, and in some cases even impossible because of the detection limit of the analytical techniques at hand/or sampling requirements (e.g. for the safety of sterilized low-acid canned foods, public health policies impose that the « probability of a non sterile unit (PNSU) » should not exceed 10^{-9} (Plug, 1987a-c), which can not be monitored. New trends with regard to the in-situ approach to evaluate thermal processes involve the development of more sensitive and rapid analytical methods.

In the physical-mathematical (eq.1.7 or 1.8) method, the calculation of the process value imposed on the food either requires the use of temperature sensors to record the time-temperature history at the critical point of the product or requires the use of theoretical solutions of heat transfer models or of (semi-) empirical methods (e.g. Ball and Olson, 1957; Hayakawa, 1970) that use the knowledge of process parameters and heat penetration parameters of the food product itself combined with knowledge on the kinetics of the safety/quality attribute under study. A critical analysis of the different mathematical procedures for evaluation and design of in-container thermal processes for foods is available (Stoforos, *et al*, 1997). Further information can also be found in a critical review about the evaluation of the integrated time-temperature effect in thermal processing of foods (Hendrickx *et al*., 1995).

A major advantage of empirical or theoretical formula methods is their predictive power for processing conditions different from those under which experiments were run. For instance, the semi-analytical method of Ball and Olson allows, once the heat penetration parameters of a product are known (f_h and j) for a given product-packaging combination, to estimate the process value achieved with a different packaging of the same product exposed to similar process conditions (Come Up Time, initial temperature at the critical point of the product, holding temperature). Moreover, this method allows to calculate the required holding time at a given holding temperature to reach a given target process value.

With regard to the semi-analytical method of Ball and Olson, which is the most commonly used in industry, the knowledge of f_h and j can be achieved in two different ways: experimental determination (CUT ideally close to 0) or extrapolation calculation between f_h and j for a given product packaging and the same product in an other packaging (different volume, shape, amount of product). In both cases, however, the f_h and j determination requires at least one heat penetration experiment. Several problems can then arise: 1- direct registration of the time-temperature profile of the product is not possible under some processing conditions:

cable thermal probes can not be used in continuous thermal processing and wireless thermal sensors disturb too much the heat transfer in the product (miniaturized wireless temperature recorders, although being an interesting idea, would probably always suffer from their lack of flexibility in the adjustment of their shape, density and thermal conductivity). 2-The critical point of a product, except for real conductive products, is almost always moving during the heating process (Whether they are discontinuous or continuous, a number of heat treatments submit products to axial or end over end rotation in order to take advantage of their convective characteristics to increase the heating rate).

Moreover, with the Ball and Olson method, the thermal impact of CUT and overshoot (this is the still increasing temperature in the product after the beginning of the cooling step) is not taken into account leading to underestimation of the real process values. No process optimization is achieved in this case but rather process safety can be evaluated. In order to take into account the thermal impact during overshoot and CUT, algorithms have been developed to calculate, all along the heat treatment, the acquired process value and anticipate the temperature history during heating and cooling while integrating the time temperature profile to obtain the resulting process value (e.g. OPTIBAR software developed by CTCPA, ENSIA and COMEUREG, France; TPRO software developed by Dr. J. Norback, University of Wisconsin). Hence, it becomes possible to anticipate, in only one run, the cooling start time in order to reach the target process value with an accuracy of 5-10%. However the use of this interesting and currently used optimization tool is limited to discontinuous thermal processes and can show limitations for convective or particulate food products submitted to rotary processes because of the permanent moving of the critical point and perturbations of convection heating induced by the probes. Beside expensive material requirements, in this last case, food containers must be strongly fixed in the retort in order to avoid damaging of thermal cable probes during rotation and several assays must be performed to choose the most unfavorable option (the longest exposure time observed at the holding temperature) leading to the desired process value. In conclusion this optimization method, which does not require the previous knowledge of f_h and j heat penetration parameters (because direct temperature measurement is performed) is not adapted to all different kinds of thermal processes and food products.

1.4 Conclusion

The physical-mathematical method (using direct temperature registration or semi empirical heat transfer models) shows limitations in the design and evaluation of more recently introduced thermal preservation processes. The possible advantage of new heating techniques such as volumic heating followed by aseptic packaging (e.g. ohmic heating followed by a bag in box aseptic packaging system) or microwave heating, could be seriously affected without establishment of adapted thermal impact evaluation techniques. In this context, development of

time temperature integrators (TTIs) that allow fast, easy and correct determination of the thermal impact on a product attribute, without any necessity to know the time temperature history of the critical point of the product, currently receives great interest.

BACKGROUND OF THE INVENTION

1 Introduction

As introduced in the previous chapter, Time Temperature Integrators are considered as a possible way to overcome the inherent disadvantages of the in-situ and the physical mathematical method for thermal process evaluation. In this chapter, some general aspects of TTIs as modern thermal process evaluation tools and a current state of the art regarding TTI development are presented. This part focuses on pasteurization and sterilization processes. TTIs to monitor quality losses during low temperature (refrigeration/freezing) storage and distribution are not discussed here.

2 Definition of a time temperature integrator

A TTI can be defined as « a small measuring device that shows a time-temperature dependent, easily, accurately and precisely measurable irreversible change that mimics the change of a target attribute undergoing the same variable temperature exposure » (Taoukis and Labuza, 1989a-b; Weng *et al.*, 1991a-b). TTIs are wireless systems. Their main advantage is the ability to quantify the integrated time-temperature impact on a target attribute without the need for information on the actual temperature history of the product. In a way, TTIs show similarity with the in-situ method because it is also a « post-facturn » method. TTIs do not provide any knowledge about time-temperature history but only show consequences of this time-temperature history on a given target attribute.

3 Criteria for a time temperature integrator

According to the TTI definition given above, a TTI is a small measuring device that should meet the following criteria:

1-For convenience, the TTI has to be inexpensive, quickly and easily prepared, easy to recover and give an accurate and user-friendly read-out.

2-The TTI should be incorporated in the food without disturbing heat transfer within the food. The presence of the TTI must not change the time temperature profile of the food, and the TTI should be exposed to the same time-temperature profile as the target attribute under investigation.

3-The TTI should quantify the impact of the process on the target attribute under study. In other words, a TTI has to meet some kinetic requirements. The temperature dependency of the rate constants of TTI and target attribute should be described by the same law (e.g. Arrhenius model, TDT model). Moreover, it can be easily shown from equation (1.7) and (1.8) that the temperature sensitivity of the rate constants (E_a-value or z-value) of the TTI and of the target attribute should be equal to assure the equality in process-values (Maesmans *et al.*, 1993, Hendrickx *et al.* 1995). As in practice it can be difficult to obtain exactly the same thermal sensitivity of the rate constant (expressed by the z value in °C) for the TTI and for the target attribute, several solutions have been suggested. 1) Van Loey *et al.* (1995a) studied in a theoretical way the allowed difference in z-value between a single component time/temperature integrator and target attribute to measure the impact of a thermal process with a given accuracy. In order to always evaluate the thermal impact in a safe way (that is by underestimating the real process impact) she proposed a method based on the use of a TTI with a z value smaller than that of the target attribute. 2) Pflug and Christensen (1980) proposed a method based on the conversion of a thermal impact with a given z value in another thermal impact determined on the basis of an other z value (in this case information on heating rate characteristics are required). 3) Maesmans *et al* (1993) have studied theoretically the possibility to calculate a thermal impact on a target attribute by using the defined thermal impacts on different TTI compounds with known z-values different from the target attribute's one: this is the Multicomponent TTI concept which has also been studied by Stoforos and Taoukis (1998). 4) Maesmans *et al.* (1994) studied the possibility to combine the use of the equivalent point method (Swartzel, 1986, Swartzel *et al.*, 1991, Sadeghi and Swartzel, 1990) and a multicomponent time-temperature integrator.

In spite of the theoretical interest of multicomponent time-temperature integrators, they show some potential limitations due to the necessity to measure the thermal impact on at least two TTI components instead of one (with single component TTI). This is not in line with the convenience mentioned in the above TTI definition. However, multicomponent time temperature integrators, although requiring a consequent development work, could remain an interesting tool to monitor process-values in case the measures of the thermal impact on the several (at least two) components would be easy and fast to perform. According to Stoforos and Taoukis (1998), the best option, when developing multi-component TTIs should be to include components with z-values below and above the one of the target attribute under concern. In case this option can not be achieved they advise to use components showing z-values above rather than below the z-value of the target attribute of interest.

As a kinetic requirement it is also important that the TTI possess an acceptable heat inactivation rate D (or k) value at the desired processing temperature. If the D-value is too low, the TTI can be (almost) totally inactivated so that the TTI response may be below the detection limit of the reading method. A too high D-value may lead to a too limited change in TTI response (too low for accurate detection of changes).

4 Classification of time temperature integrators

TTIs can be classified in terms of working principle, type of response, origin, application in the food material and location in the food as shown in figure 2.1 (Hendrickx *et al.*, 1993, 1995). Depending on the working principles, TTIs can be subdivided into biological (microbiological and enzymic), chemical and physical systems. Single component TTIs (with a z or E_a-value equal to the target z or E_a-value) are easier to use than multicomponent TTIs and hence much more desirable. Multicomponent TTIs could, however, be an alternative if no suitable single component TTI is available. Concerning the origin of TTIs, extrinsic and intrinsic TTIs can be distinguished. An extrinsic TTI is incorporated into the food whereas intrinsic TTIs are present in the food and represent the behavior of another food aspect. With regard to the application of the TTI in the food product three approaches can be distinguished: dispersed, permeable or isolated. In dispersed systems, the TTI (extrinsic or intrinsic) is homogeneously distributed throughout the food, allowing evaluation of the volume-average impact of a process. Besides dispersion of an extrinsic TTI in the food, extrinsic TTIs may be permeable (permitting some diffusion of food components into the TTI) or isolated. All three approaches can be the basis for single point evaluations on the process impact at a specific location within the food.

In the case of dispersed and permeable TTIs, temperature is no longer the only factor that can influence the TTI response. Intrinsic properties of the food such as salinity, pH etc...can also have an influence on the TTI kinetics. These intrinsic properties should be taken into account when developing a dispersed or permeable TTI. Hence, it remains possible to calculate a process value using equations (1.11) or (1.12) because the TTI and the target attribute show the same inactivation kinetics under the same environmental conditions of the sterilization process.

Encapsulated TTIs have been proposed to avoid the influence of the food environment on the kinetic behavior of the TTI. In this case, the TTI is completely isolated from the food environment by embedding it in an inert carrier material such as glass, plastic or metal. Hence, temperature becomes the only influencing factor. It is necessary to choose a TTI carrier with a thermal resistivity as small as possible in order to ascertain that the observed TTI response is due to the food product heating and not limited by the heating of the carrier material. For instance, use of small hermetically sealed or screwed highly conductive metal carrier systems guarantees the elimination of any influence of the environment other than temperature and allows its incorporation into a real food product. Some of the required properties that any TTI-carrier system should meet have been discussed (Maesmans et al., 1993):

-thermal diffusivity as close as possible to the food's one.

-In the case of particulated foods heated in a rotary process (e.g. end over end rotation) or a continuous process (e.g. aseptic processing), the TTI carrier should possess (once filled with TTI) the same density and, if possible, shape as the critical particle in order to mimic as close as

possible the movement of the particle during the process. It is clear that for each liquid/particulates food a previous study of thermal conductivity, shape, density will have to be performed in order to determine the critical particle that will have to be "mimicked" by the carrier.

-The TTI carrier should possess an acceptable mechanical resistance in order to keep its shape when submitted to mechanical stress (e.g. particulated foods pumped during volumetric heating processes or surface scraped heat exchanger).

-The carrier material should be adapted to the applied heating process (e.g. avoid the use of metallic carrier with ohmic heating or with microwave heating).

-Other carrier characteristics such as convenience in recovering, chemical compatibility with the processed food, price, are also important.

Table 2.1 and 2.2 give a state of the art of TTIs.

Microbial TTIs

As it can be seen in tables 2.1 and 2.2, Bacillus sp. are the most commonly used for development of microbial TTIs in the food and pharmaceutical industry. One can distinguish two kinds of microbial TTIs:

-Count reduction systems which allow, according to equations (1.11) or (1.12) to calculate a process value.
-Survivor/Kill systems that only allow to identify if the thermal process was sufficient (no growth) or not (growth) to kill all microorganisms present in the food.

The major disadvantage of any microbial monitoring system is the time required to perform the assay. The long incubation time (up to several days) and read-out of the system does not allow for rapid intervention upon any kind of (systematic) failure or process deviation. Quantitative microbiology has to be performed by skilled manpower (Pflug and Smith, 1977) and the analytical precision of currently available techniques is rather low (Jason, 1983). Heat resistance determination of spores requires thorough calibration. This step to determine the killing power of a given heat treatment is difficult to achieve (Pflug and Odlaug, 1986). The inherent limitations of microbiological detection methods in determining the efficacy of thermal processing, together with the time and expense associated with these methods, have prompted the investigation of alternatives (Mulley et al., 1975b).

Protein-based TTIs

The potential of protein based systems, in particular enzyme based systems, is receiving considerable interest. The relative easiness of read-out and handling of enzymic systems offers significant advantage over microbial TTIs. Moreover, the range of heat denaturation kinetics of proteins includes the typical values for both safety and quality aspects. In general, enzyme inactivation is characterized by a z value in the range from 6.5°C to 55°C, whereas the z-value of the thermal inactivation of vegetative cells and spores is in the range 4.5° to 12°C and for quality aspects (e.g. color, texture, flavor, vitamins) in the range from 25°C to 45 °C (Lund, 1977). These characteristics are of interest with regard to safety considerations because the design of sterilization processes for low acid canned food (LACF) are directed to the destruction of spores of proteolytic strains of *Clostridium botulinum* with a z-value of 10°C. For the evaluation of safety of pasteurization processes several microorganisms with z-values ranging from 5°C to 12°C have been advanced for use as reference organism because depending on the type of food, intended shelf life, different microorganisms might be the main cause of poisoning. In enzyme based monitoring systems, often the enzymic activity remaining after the heat treatment is assayed to determine the thermal impact, although other properties, such as the heat of enzyme deterioration, can be determined instead (Use of Differential Scanning Calorimetry). TTI based on thermostable enzymes show interesting features:

-They can be small (smaller than 0.5 cm^3).
-They are relatively low in price.
-They are easier to prepare than microbiological TTIs.
-Both residual activity and residual enthalpy can be measured rapidly and accurately and hence be used as TTIs response reading methods. The response is then obtained in few minutes instead of several days with microbiological TTIs.
-Their thermostability allows them to be used in pasteurization as well as in sterilization processes.
-Their heat inactivation kinetics can be manipulated in different ways in order to reach the desired thermal sensitivity (figure 2.2).

As mentioned in tables 2.1 and 2.2, α-amylases from different *Bacillus* species have been most frequently studied. The KULeuven Laboratory of Food Technology focused during the last ten years on the study of α-amylases as potential enzymic TTIs for pasteurization and sterilization processes. The feasibility of using *Bacillus licheniformis* α-amylases covalently immobilized on glass beads or *Bacillus amyloliquefaciens* α-amylases in the presence of polyols including carbohydrates, as TTIs, has been evaluated (De Cordt *et al.*, 1992a-b, 1993, 1994). Potential TTIs based on *Bacillus subtilis* or *Bacillus amyloliquefaciens* α-amylase to monitor safety of pasteurization and sterilization processes have been developed (Van Loey *et al.*,1996,1997a-b)

on the basis of residual enthalpy reading methods on moisture controlled enzymic systems or on enzyme/carboydrates mixtures in solution. Potential TTIs based on *Bacillus amyloliquefaciens* at reduced moisture contents and residual activity or enthalpy reading have been described by Haentjens *et al.* (1998) and Guiavarc'h *et al.* (2002). Although these α-amylase based TTI systems are valuable research tools, the need of an enzymic system accurate inexpensive and convenient to use for industrial applications is still a question at this moment .

Chemical TTIs

To overcome inherent disadvantages of microbiological TTIs, chemical systems based on a purely chemical response towards time and temperature have been proposed (table (2.1) and (2.2)). The concept of a chemical TTI for evaluation of thermal process impact was advocated more than 25 years ago (Mulley, 1975a-b). Because of their great flexibility of handling and high analytical precision in the detection of chemical reactions, chemical TTIs are considered as promising tools for the evaluation of a thermal process (Danielson, 1982). The crucial deficiency of chemical TTIs, however, is that no reaction has been identified in the open literature on heat treatment of foods that feature the activation energy (or z-value) required for monitoring food safety, and only few are available that can be used to follow the deterioration of other quality attributes.

Physical TTIs

Witonsky (1977) and Swartzel *et al.* (1991) studied TTIs based on a physical working principle.

5 What is commercially available?

An overview of commercially available indicators for thermal process validation is given by Selman (1995). Different societies such as 3M industrial tapes and adhesives, Manchester, UK; Color Therm., Surrey, UK; Spiring Earnest, Germany; Albert Browne Ltd, Leicester, UK; PyMaH Corp., Flemington, N.J, USA, market different kinds of indicators. For instance, 3M developed the « Monitor Mark (TM) High Temperature Threshold Indicator », which indicates visually the cumulative exposure time above a predetermined temperature in shipping (available in button, label and tag formats). However, none of these indicators can provide relevant information on the in-pack thermal efficiency of a process. Therefore, they are not really appropriate to monitor thermal processes in the food industry. Three categories can be distinguished:

-The first category indicates only whether a pre-set temperature has been reached and/or exceeded.

-The second category reacts after exposure for a fixed period at a constant temperature.

-The third category integrates the full temperature history but can only be placed on the outside of containers.

These indicators are often based on the color change of a thermo sensitive ink. They are extensively used in the drug industry (e.g. sterilization of medical tools). Although the present work focuses on heat treatments, it should be indicated that many societies are involved in the development of indicators for shelf life determination and storage management (Cold chain monitoring) (e.g. Cox technologies, Belmont, USA; 3M industrial tapes and adhesives, Manchester, UK), which are useful for food products. However, for heating processes, there is a pressing need for new systems that allow quick, correct and easy quantification of the thermal process impact, especially in terms of food safety.

6 Conclusion

According to the above mentioned need and taking into account the merits and limitations of different kinds of TTIs, the KULeuven Laboratory of Food Technology decided to give the priority to the following research approach:

« Development of SINGLE or MULTI component(s), ISOLATED, EXTRINSIC, ENZYMIC TTIs » for safety evaluation of sterilization processes.

As mentioned in 2.4, such potentially interesting enzymic systems for the evaluation of in pack thermal efficiency of pasteurization processes and sterilization processes have been developed. Although these α-amylase based TTI systems are valuable research tools, the need of an enzymic system that is accurate, inexpensive and convenient to use for the monitoring of industrial sterilization processes is still existing at this moment. Indeed the most interesting enzymic TTI available (at the laboratory scale) up to now was a TTI based on *Bacillus subtilis* α-amylase equilibrated at a 76% equilibrium relative humidity (Van Loey *et al.* 1996, 1997). But this system shows the following limitations:

-the range of process values that can be investigated is too low (max up to 14 min) with regard to large process values observed in the industry (up to more than 60 min).

-These TTIs are difficult and time consuming to prepare (3 weeks).
-They require a larger amount of pure dehydrated enzyme (approx 10 mg per TTI).

-Residual enthalpy of denaturation, requiring expensive Differential Scanning Calorimetry equipment, is used as a response property. Residual activity could also be used as a response property but protein aggregation phenomena cause difficulties to solubilize the enzyme before activity reading.

-They are not stable during storage between preparation and use.
-They are not stable during storage between heating and reading.
-Depending on the commercial batch of enzyme used, different heat denaturation kinetic parameters (D-values and z-value) may be observed.

To overcome these limitations, an efficient and standardized enzymic TTI preparation procedure was developed, which allows to use enzymes as accurate, inexpensive and convenient time-temperature integrators, when showing first order inactivation, for the monitoring of thermal (sterilization) process impacts. This procedure was applied for the first time to *Bacillus licheniformis* and *Bacillus subtilis* α-amylases.
The original idea of this procedure was to use an inert filler made of glass beads to avoid the aggregations phenomena mentioned above. Several advantages follow from this idea. The main advantage is that enzymes are efficiently spread all over the surfaces of the beads to avoid aggregation phenomena during heating that leads, after heating, to a protein network impossible to solubilize for subsequent activity measurement. Hence the glass beads are not only used as a simple adsorption surface allowing to fix the enzyme, they are also used as an inert filler separating the enzyme molecules from each other.
An important consequence of this is that a very small amount of enzyme can be used to prepare the TTI and that this small amount can easily be solubilized for residual activity determination.
In the following part the original preparation procedure, when applied to a *Bacillus licheniformis* α-amylase will be firstly described. The key steps of this procedure will be explained with help of few experimental results (see annexes). Also experiments related to necessary heat inactivation kinetic aspects will be introduced. Finally, the results obtained with an enzymic TTI based on *Bacillus licheniformis* α-amylase and to a less extent with an enzymic TTI prepared with *Bacillus subtilis* α-amylase (both prepared following the original preparation procedure) will be presented and claims relative to the originality of the present invention and of its exploitation will be introduced.

SUMMARY OF THE INVENTION

The objective of the present patent is to protect the TTI preparation procedure (part 3.1) described above, whatever the enzyme of concern in this preparation procedure.
This procedure seems crucial in obtaining accurate and reproducible TTIs with a z-value in the range 8-16 °C allowing the determination of process values with an acceptable accuracy.

The preparation procedure (part 3.1) allows to obtain the following original product:

"A mix = a strongly dehydrated enzyme adsorbed at the surface of non porous glass beads with stabilizing substances (sucrose and salts). This mix is packed in a hermetically sealed container ,e.g. small DSC capsules coated by a hermetical silicone layer."

The first key idea of the present TTI preparation procedure is the use of an inert filler, in our case glass beads of approximately 0.250 mm diameter. The main advantage of this inert filler is to avoid aggregation phenomenon during heating that lead, after heating, to a protein network impossible to solubilize for subsequent activity measurement. Hence the glass beads are not only used as a simple adsorption surface allowing to stabilize the enzyme, they are also used as an inert filler allowing a convenient read out of the system.
An important consequence of this is that a very small amount of enzyme can be used to prepare the TTI and that this small amount can easily be solubilized for the determination of the residual activity.

The second key idea of the present TTI preparation procedure is the use of an extra-dehydration step, inside an oven at a temperature well above the room temperature, of the mix contained inside the DSC pans followed by an immediate sealing of the capsules and an entrapment inside silicone. From these three steps (extra-dehydration, immediate sealing, entrapment in silicone) follow several advantages:
-Achievement of a good and similar control of the level of dehydration of the mix inside each capsule and subsequently achievement of a good level of accuracy of the TTI response (TTI show reproducible and stable constant z-value).
-Achievement of a high level of thermostability allowing to determine thermal impact at temperature higher than 100°C.
-Achievement of an interesting ability to store the TTI between its preparation and its use and between its use and its reading.

Moreover, the packed mix is easy to remove rapidly from DSC capsules coated with silicone.

The enzyme of this packed mix shows the following interesting functional characteristics:

-After determination of its heat inactivation parameters (D-values and z-value), it allows to monitor process-values at high temperature exposure.
For instance, when using *Bacillus licheniformis* α-amylase, it is possible to monitor process-values up to around 70 min in a temperature range of 100-140°C.
-It is easy, by addition of 1 ml of water on the mix contained in one capsule and rapid mixing, to obtain rapidly its total desorption from the surface of the beads.
-It shows a great stability (several months) of its D-values and z-value, when the capsules coated with silicone are stored at ambient temperature.
-It shows an acceptable stability (several days in the case of BLA) of its residual activity after heating treatment.

DETAILLED DESCRIPTION OF THE INVENTION

1. MATERIALS AND METHODS

1.1 Detailed preparation of the TTI

1.1.1 Standardization of the enzymes' environment

The two following steps are required:

-Standardization of the liquid environment of the enzymes and standardization of the concentrations of these enzymes in the liquid environment. The standardized liquid environment has to contain sucrose and sodium chloride as stabilizers.
-Mix of the standardized solution with a non-porous inert filler (e.g. glass beads) resulting in adsorption of the enzymes at the surface of the inert filler. In case glass beads are used as an inert filler, the required volume of standardized solution is the exact volume necessary to fill in the spaces between the glass beads.

An example of standardization is introduced below when using a *Bacillus licheniformis* α-amylase and glass beads for the preparation of an enzyme-based TTI:

1-Take a sample of *Bacillus licheniformis* α-amylase (BLA) solution.

2-Determine the protein content (in mg per ml) of the sample using a Bicinchoninic Acid Protein determination Kit BCA1 of Sigma. If unknown, determine also the iso-electric point (pI) and the molecular weight (MW) by using an isofocusing method and SDS-PAGE method respectively.

3-Prepare a buffer corresponding to the pI of the enzyme.

For *Bacillus licheniformis* α-amylase prepare 1000 ml of a 0.005M bis-tris buffer, pH=6.9 as follow:

-Dissolve 1.046g of bis-tris base (MW=209.2) in approx. 900 ml of distilled water at 25°C.

-Add 2.58 g of dehydrated calcium chloride (CaCl2, 2H2O).

-Add 15.24 g of sodium chloride (NaCl).

-add 20 g of sucrose.

-add 1.27 g of potassium chloride (KCl).

-Titrate to pH 6.9 with HCl 0.1N at 25°C.

-Make up volume to 1000 ml with distilled water (Buffer will be pH=6.9 at 25°C).

4-On the basis of the protein content obtained in 2, dilute a sample of the BLA solution with the buffer in order to reach a protein concentration of 1mg/ml. (Do the same whatever the enzyme used).

5-Ultrafiltrate 9 ml of the solution obtained in point 4 by using MACROSEP 3000, 10000 or 30000 kDaltons centrifugal devices (Pall Life Sciences, USA). In case other enzymes with different Molecular Weight than *Bacillus licheniformis* α-amylase are used, it is of course necessary to adapt the filter to the molecular weight). The centrifugation is performed in 2 steps:

-6000 rpm, 120 min at 4°C for ultra-filtration.

-6000 rpm, 1 min at 4°C for the recovery of the retentat.

6-Complete the retentat with the buffer prepared in 3 in order to obtain 5 folds the volume of the BLA solution taken initially and contained in the 9 ml ultra-filtered in point 5. This step is explained in Annex I).

7-Fill 1.5 ml eppendorf cups with 1.35g of glass beads of approx 0.25 mm diameter and add 0.281 ml of the standardized enzyme solution obtained in point 6 (this volume allows to just fill in spaces between glass beads). On the basis of the example mentioned in the Annex I, it would be possible to fill 1.385/0.281=4.92 eppendorf cups.

8-Seal the tip of a glass capillary with a flame and use this capillary, once cooled, to mix correctly the beads and the solution obtained in point 6 to achieve a clear brown homogeneous color.

1.1. 2 Dehydration of the mix

A strong level of dehydration is achieved in two steps:

-Freeze drying allowing to remove almost all the water contained in the mix.
-Extra-dehydration of the mix by evaporation drying in an oven at a temperature well above room temperature up to constant weight (control with a balance showing an sensitivity of 0.01 mg).

For instance, the dehydration procedure applied to the mix achieved in step 8 of the example introduced in 3.1.1 is described below:

9-Dehydrate by Freeze drying the content of the eppendorf cups (e.g. by use of a Christ (Switzerland) freeze drier) with the following freeze-drying parameters:

Safety pressure heating: 10 mbar.

Pressure main drying: 0.180 mbar.
Ice condenser: -82°C
Temperature of the shelve during main drying: +4°C.
Freeze-drying time: 20 hours.

This step allows to remove the water between beads under non-denaturing conditions. While the water is eliminated by sublimation, the enzyme, the ions and the sucrose accumulate at the surface of the glass beads. At the end of the freeze drying, these compounds coat the beads and act as a weak cement between them. After release of the vacuum, a 3-D network made of glass beads, enzyme, salts, sucrose and air is obtained.

10-Recuperate the eppendorf cups. Their content consists of a solid block because proteins, sugars and salts, act as cement between the beads. With help of a stainless-steel spatula, a homogeneous powder is obtained. This powder (we will also call it "mix" in the following lines) is made of beads of which the surface is covered by a thin layer of enzymes and stabilizers (sucrose and salts).
The main advantage is that proteins are sufficiently spread over the surfaces of the beads to avoid aggregation phenomenon during heating that lead, after heating, to a protein network impossible to solubilize for subsequent activity measurement. Hence the glass beads are not only used as a simple adsorption surface allowing to stabilize the enzyme, they are also used as an inert filler avoiding aggregation and enhancing protein solubilization after thermal treatment.

An important consequence of this is also that a very small amount of enzyme can be used to prepare the TTI.

11-Mix the content of the eppendorf cups together inside a plastic tube to achieve a homogeneous mix and place the latter inside clean eppendorf cups.

12-Store the eppendorf cups containing the homogeneous mix inside a dessicator containing P_2O_5 (But encapsulation can also be performed immediately).

13-Equip pan covers with O-rings (Perkin-Elmer, kit 0319-0029). Place them on a glass plate with the O-ring face visible. Let dry the whole at 102°C during 15 min in an oven. Then, remove the plate containing the covers from the oven and wait for cooling. Once cooling is achieved, turn each cover (O-ring face not visible).

14-Fill the pans of 60µl Large Volume Capsules (Perkin-Elmer, kit 0319-0029) with 40 to 60 mg of mix. This step is performed extremely rapidly using a spatula. Some negligible loss of mix may occur here (mix falling outside the pans). However, according to point 7, it should be possible to fill approximately 100 pans starting from one centrifugation.

15-Further-dehydrate the freeze dried mix by placing the filled pans at 102°C during 1 hour inside an oven. It was possible to perform an extra-dehydration of 1 hour in an oven at 102°C with BLA, without any inactivation of the enzyme (here we took advantage of the natural thermostability of the enzyme). When applying the same preparation procedure to another enzyme it would be necessary to check if this enzyme is inactivated or not. From a basic point of view, whatever the enzyme contained in the mix obtained after freeze-drying, this enzyme should be sufficiently stabilized to be dried in an oven at a temperature higher than the room temperature. The experimental evidences that motivated the further-dehydration of the freeze dried mix are detailed in Annex II.

1.1. 3 Encapsulation of the dehydrated mix avoiding moisture entrance

The objective is to encapsulate the dehydrated mix in a small and hermetically closed container. It is necessary to avoid that the dehydrated mix takes up atmospheric moisture during encapsulation.

For instance, the encapsulation procedure (using DSC capsules as containers) applied to the mix dehydrated in step 15 of the example introduced in 3.1.2 is described below:

16-After 1 hour of drying, remove the pans from the oven one by one and seal rapidly the covers on them (less than 10 seconds is easily achieved) according to Perkin-Elmer recommendations. Close the oven door between each sealing.

17-let the capsules containing the mix cool down to ambient temperature and store the whole inside a dessicator containing some phosphopentoxide (P_2O_5).

18-Entrap the capsules, containing the dried homogeneous mix, inside Sylgard 184 Silicone elastomer (Dow Corning Chemical, Belgium) according to the following procedure:

-dispose the capsules in rows on a glass plate in such a way that each row is separated from another row by a distance of 0.5 cm minimum. The cover of the capsules has to be in contact with the plate.

-prepare Sylgard 184 silicone following the recommendations of Dow Corning Chemical (10 part of Part A with 1 part of B (curing agent)).

-pour the liquid silicone on the plate containing the capsules up to cover the capsules with 1 mm of silicone.

-let dry at 102°C inside an oven during 20 min.

-recuperate the plate with the capsules and the solidified silicon and let cool at ambient temperature.

-with a cutter, cut silicone around each capsule to obtain a squared piece of silicone (1 cm of side) containing the capsule.

-turn each piece of silicone containing a capsule and, with help of a small glass capillary add a thin layer (0.5 mm) of liquid silicone on the face of the capsule non-protected by silicone.

-dry at 102°C in an oven during 15 min.

-recuperate the pieces of silicone containing the capsules and remove the silicone corners with a cutter in order to obtain an octogonal shape. (a small punch can also be used).

The experimental evidences of the necessity to coat the capsules with silicone are detailed in Annex III.

1.2 Measurement of the TTIs' responses: Process-value determination

Residual activity of the enzymes used to prepare the TTIs is used as a response property.

For instance, when using a TTI based on a *Bacillus licheniformis* α-amylase , the α-amylase activity is measured spectrophotometrically at 30°C according to procedure no.577 of Sigma

Diagnostics. This procedure is based on the final release of para-nitrophenol, which absorbs maximally at 405 nm.

Activities are expressed in terms of the change in optical density per min, calculated by linear regression from a plot of the absorption versus reaction time. The activity was deduced from the slope between 2 and 4 minutes. A temperature of 30°C was used to perform the activity assay.

Because residual activity was used as response property, a rapid and efficient way to put the enzyme from the mix in solution was necessary. The following method appeared to be efficient:

1-Dispose the same number of eppendorf cups than capsules to be read on a rack. Number each of them with a marker.
2-Put an empty 1.5 ml eppendorf cup with safe lock on the plate of an accurate balance with a sensitivity of 0.0001g. Tare.
3-Open a capsule by double lateral compression with help of a simple small bench vice. During the compressions, the capsule is kept between 2 fingers. In this way, less than 10 seconds are required to open a capsule.

4-collect some mix from the pan of the capsule inside the eppendorf cup. Close the latter, put it on the balance. The value displayed by the balance corresponds to the amount of mix. This amount should be between 15 mg and 60 mg. Note it.
Redo step 2 to 4 for every capsule.
5-Solubilize the enzyme as follows:
-add 1 ml of distilled water in the corresponding eppendorf cup containing an amount of mix from the capsule.
-close the eppendorf cup.
-mix the eppendorf in a radial way with help of a vortex (2000 rpm 30 seconds).
-mix the eppendorf axially with hand during 30 seconds (end over end rotation).
-pipet 1 ml of Amylase Reagent and 0.020 ml of the solution contained inside the eppendorf cup in a cuvet. Mix immediately by inversion and incubate at desired temperature during 2 minutes.
-Determine the slope of the absorbance versus time curve between 2 and 4 minutes.
Repeat step 5 for every capsule.
6-from each slope, deduce a residual activity A in Sigma Units/liter following Sigma kit recommendations. If the observed residual activity is higher than 2000 Sigma units/liter, dilute the enzymic solution from the eppendorf in order to obtain a lower activity and repeat the measurement.
7-With the observed residual activity A and the initial activity A0 corresponding to the same amount of non-heated mix from an non-heated capsule (everything can be e.g. calculated on the basis of 50 mg of mix) and the $D_{121.1°C}$-value of the TTI , calculate the process value F_{TTI} using equation 1.11..

1.3 Thermal characteristics of the TTI

1.3.1 Determination of the TTIs' heat inactivation kinetic parameters (D-values and z-value) under isothermal conditions

1.3.1.1 Use of the classical two step regression method

Under isothermal conditions, a first order reaction can be written as equation (3.1):

$$\ln\frac{X}{X_0} = -k \cdot t \qquad (3.1)$$

X0= initial response value (e.g. initial enzyme activity A_0 or initial enthalpy of denaturation H_0).

X= response value after heating treatment (e.g. residual enzymic activity A or residual enthalpy of denaturation H) .
t= exposure time (min)
k= inactivation-denaturation rate constant (min-1)

or in the thermal death time terminology as equation (3.2)

$$\log\frac{X}{X_0} = -\frac{1}{D} \cdot t \qquad (3.2)$$

where D is the decimal reduction time (time for one log reduction of the response at a given temperature). The D-value can be calculated from the slope of the linear regression line of log (X/X_0) versus time.

On the basis of equation (3.3) (Bigelow 1921), the thermal sensitivity of the decimal reduction time, expressed by the z-value (°C), can be estimated by linear regression of log D-values versus corresponding temperatures.

$$D_{T_2} = D_{T_1} \cdot 10^{\left(\frac{T_1 - T_2}{z}\right)} \qquad (3.3)$$

It is proclaimed in the literature that when determining the slope of a first order reaction the best accuracy is obtained by placing the experimental points at the two borders of the experimental domain (Goupy,1988). In our case, for each exposure temperature investigated, the experimental domain corresponded to the time interval between time 0 (no denaturation) and the maximal exposure time corresponding to the detection limit of the reading method (in our case reading of the residual activity of the enzyme). On the basis of our experiments, it was demonstrated that the inactivations kinetics of *Bacillus licheniformis* α-amylase at low moisture content followed first order kinetics. Hence, it is possible to use only 2 exposure times (close to the borders of the experimental domain) per investigated temperature (time 0 excluded) to determine the D-values at each exposure temperature. Accuracy was also increased by using 4 points per time-temperature combination. Isothermal heating of the sample was carried out by immersing simultaneously several TTIs inside an oil bath (Grant, England) with silicone oil from Fluka (ref 85415). Cooling was performed in ice-water at 0°C.

1.3.1.2 Use of non-linear regression (one step approach)

An advantage of the above mentioned two step method is that it allows to check graphically the linearity of the inactivation-denaturation curves (of course when different heating times are used). However, since superiority of a global fit is proclaimed throughout literature (Cohen and Saguy 1985 a-b; Haralampu and others 1985; Myers 1990; Van Boekel 1996), in the present study , the heat inactivation-denaturation kinetic parameters D_{Tref} and z were also estimated in a global fit using non linear regression analysis (SAS 1982) on equation 3.4 (deduced from equations 3.2 and 3.3).

$$\frac{X}{X_o} = 10^{-\frac{t}{D_{Tref}} \cdot 10^{\left(\frac{T-T_{ref}}{z}\right)}} \qquad (3.4)$$

where X_o and X correspond to initial and residual activities observed after isothermal heating.

1.3.2 Validation of the TTI under non-isothermal conditions

Since real thermal processing conditions involve non-steady state temperature conditions, it was necessary to evaluate the performance of the TTI system under variable temperature conditions. Various non-isothermal profiles were generated by placing the TTIs inside 2.5 cm diameter silicon spheres (Dow Corning Sylgard 184 Silicone Elastomer, Belgium) as described in fig 3.1. Thermocouples were prepared using 0.081 mm diameter copper-constantan wires (Omega,

Belgium) and Ellab connectors and were connected to a CMC-92 data acquisition system (TR9216, Ellab, Denmark). An accuracy of 0.1°C was obtained with each thermocouple by comparison with a reference quartz temperature sensor (Testo, Belgium). Spheres were placed simultaneously in an oil-bath (Grant, England) at 123.2°C and time-temperature recording was started at the moment of immersion. Process values Ft-T corresponding to each sphere were calculated by means of equation 1.8 using a recording time-step of 15 s, a reference temperature of 121.1°C and a z-value equal to the z-value of the TTI obtained under isothermal conditions. Cooling was performed by immersion in water at 0°C in order to obtain a process values in the range 3-60 min. The process-values achieved with the TTI (equation 1.11) were plotted versus the process-values achieved by the physical-mathematical method (equation 1.8), in order to validate the ability of the enzymic system to integrate correctly time and temperature under dynamic conditions.

1.3.3 Calculation of the process value on the basis of the TTI response when the z-value of the TTI is different from the z-value of the target attribute:

The final goal of the present invention is to put at the disposal of the food industry an efficient tool for monitoring thermal process impacts on a given target attribute of a given product.

In case the target attribute under study would show a z-value different from the one of the TTIs obtained following the above described TTI preparation, it is obvious that deviations between process-value deduced from the TTI response and the actual process-value concerning the target attribute under study may become more or less important depending on the actual temperature history inside the product during the thermal process. This temperature history will vary depending on the thermal characteristics of the product (heat penetration parameters) and on the characteristics of the process (holding time, holding Temperature, come up time,come down time). Two different methods can then be used to calculate a correction factor to apply to the process-value derived from the response of the TTI:

-the first method, concerning a single component TTI showing a z value different from the one of the target attribute under interest, consist in integrating the time-temperature profile (measured or simulated with a finite difference heat transfer model on infinite cylinder) undergone by the product with the z-value of the target attribute under study and the z-value of the TTIs at hand. The difference in corresponding process values ${}^{z\text{Target}}F_{T\text{ref}}$ and ${}^{z\text{TTI}}F_{T\text{ref}}$ correspond to a correction factor to apply to any TTI responses for the product and the process under consideration. This method is better described in Annex IV.

-the second method would consists of using the already known multi-component TTI concept, mentioned above. This method is described in annex V where a small part of the results of a thorough theoretical study of the potentials of three different multi-component TTIs is introduced.

1.3.4 Stability of the TTI during storage

As biosensors, the TTIs prepared according to the above described procedure may be sensitive to storage time and conditions. In the scope of an industrial use, these TTIs have to be stable

between their preparation and their use and between their heating and the reading of their response. We investigated the influence of storage time under atmospheric conditions at ambient temperature (15-25°C).

2 RESULTS

2.1 Determination of heat inactivation kinetic parameters under isothermal conditions

Figures 4.1 and 4.2 describe respectively the heat inactivation kinetics of a TTI made with *Bacillus licheniformis* α-amylase (BLA) and a TTI made with *Bacillus subtilis* α-amylase (BSA) following the above described procedure. (In annex VI, results achieved with a purified Pectine Methym Esterase (PME) are also introduced).

Tables 4.1 and 4.2 present the D-values and z-values and their associated standard error of regression (determined by linear regression) respectively for TTIs prepared with a mix based on BLA and TTI prepared with a mix based on BSA. Both mixes show standard errors of regression lower than 6% of their D-values and their z-value (except for the mix based on BLA which shows a standard error of regression of 7.6% of its D-value at 127.2°C). A D121.1-value of 45.77 min was deduced from equation 5.

2.2 Validation under non-isothermal conditions of the TTIs prepared with a mix based on BLA.

Figure 4.4, 4.5 and 4.6 introduce the following:

a-Relation between F_{TTI} and $F_{t\text{-}T}$ corresponding to the same time-temperature profiles and calculated with the z-value of the TTI.

b-Relation between F_{TTI} and $F_{t\text{-}T}$ corresponding to the same time-temperature profile and calculated with a z-value equal to 10°C.

c-Relation between $F_{t\text{-}T}$ calculated with the z-value of the TTI and $F_{t\text{-}T}$ calculated with a z-value equal to 10°C.

d- Superposition of b and c.

In figure 4, the TTI kinetic parameters are deduced from a two step linear regression method on the isothermal inactivation data (equation 3.2 and 3.3).

In figure 5, the TTI kinetic parameters are deduced from a single step method (non-linear regression : equation 3.4) on the isothermal inactivation data.

In figure 6, the TTI kinetic parameters are deduced from a single step method (non-linear regression : equation 4.1) on the non-isothermal inactivation data.

$$\frac{X}{X_0} = 10^{-\int_0^t \frac{dt}{D_{ref}} 10^{\left(\frac{T - T_{ref}}{z}\right)}} \qquad (4.1)$$

For case a and b, the absolute percentage error in process value estimation by the TTI was calculated with equation 4.2:

$$|\%\ \text{error}\ | = \left|\frac{F_{t-T} - F_{TTI}}{F_{t-T}}\right| \times 100 \qquad (4.2)$$

For case c, the absolute percentage error between $F_{t,T}$ calculated with the z-value of the TTI and $F_{t,T}$ calculated with a z-value equal to 10°C, was derived from equation 4.3:

$$|\%\ \text{error}\ | = \left|\frac{F_{t-T(z=10\ °C)} - F_{t-T(zTTI)}}{F_{t-T(z=10\ °C)}}\right| \times 100 \qquad (4.3)$$

Table 4.3 summarizes the kinetic parameters and the absolute average % error achieved when using the TTI based on a mix prepared with BLA. Whatever the set of kinetic parameters used, the TTIs allow the measurement of process-values in the range 0-60 min with an absolute average % error not greater than 13.47%. Also correlations observed between F_{TTI} and $F_{t,T}$ are definitively higher (0.98) than the correlation coefficients observed with the system based on a mix equilibrated at 48% ERH (0.90). This strong improvement of the correlation is due to the high and similar level of dehydration induced by the sealing of the capsules performed immediately after the drying step in the oven.

2.3 Stability of the TTI during storage

2.3.1 Influence of storage time on the TTI response between heating and reading

Table 4.4 introduces the evolution of the TTI-response versus storage time at ambient temperature and under atmospheric conditions. The values correspond to a TTI based on BLA prepared as described in the example introduced in part 3.1. Process-values derived from TTIs submitted simultaneously to the same heat treatments and read at different moments are listed. The initial enzymic activity used to perform the calculations is the initial activity deduced from 3 non heated TTIs at Day 0.

An interesting stability (less than 8% decay) of the process value derived from the TTIs can be observed up to 6 days after the heat treatment. This is due to a small increase of the observed residual activity with storage time (4.9% for treatment A, 4.4% for treatment B and 5.5% for treatment C, between Day 0 and day 6).

2.3.2 Influence of storage time between preparation and use

Due to the freeze-drying step and the drying step (102°C in an oven) included in the preparation procedure of the TTI, the latter is totally dehydrated and was observed to show a good stability during several months. Heat inactivation parameters (D-values and z-values) were constant for several months.

EXAMPLE I

Explanations of step 6 of the preparation procedure.

For instance: in 9 ml of the ultra-filtered enzyme solution obtained after having diluted a sample of the T120L bottle 32.42 times in order to obtain 1 mg/ml of proteins, we have 0.277 ml of enzymic solution taken in the T120l bottle. In order to dilute 5 times this volume, we have then to reach a final volume of 0.277*5=1.385 ml starting from the retentat. In case the retentat has a volume of 0.6 ml it will therefore be necessary to add 0.785 ml of buffer in order to reach 1.385 ml.

NB: A specific activity of *Bacillus licheniformis* α-amylases from three different batches of T120L was observed as being 37.33 Sigma Units/mg with a standard deviation of 3.31 Sigma Units/mg. This low standard deviation allows to use the same (5 folds) level of dilution in order to finally achieve an interesting initial activity per mass unit of TTI, that is an initial activity allowing to simplify the reading procedure of the TTI response after heating. However, in case a very different specific activity would be observed in another commercial batch of the same enzyme or with another enzyme, it would be necessary to adapt the dilution steps accordingly.

EXAMPLE II

The experimental evidence that motivates the further-dehydration (step 15) of the freeze-dried mix.

This step is crucial because it allows to limit variations of atmospheric moisture uptake by the mix inside the pans before subsequent rapid sealing. When sealing is performed rapidly at the output of the oven, the mix contained in each pan is equally dehydrated and this is a key point to achieve a good accuracy an reproducibility of the enzyme inactivation. Three experiments were conducted to achieve a better understanding of the moisture sorption behavior of the mix at the atmospheric conditions.

In the first experiment, sorption isotherms were determined for three different matrices. The first matrix was made of a freeze-dried mix of glass beads mixed with BLA solution containing sucrose and salts as stabilizers. The second matrix was made of a freeze-dried mix of glass beads and BLA solution previously dialysed against distilled water to remove stabilizers (sucrose and salts). The third matrix was glass beads alone.

Figure annex II.1 shows a comparison of the sorption isotherms obtained at 4°C. It is clear that moisture uptake is almost totally controled by the stabilizing substances (sucrose and salts) if not removed. In the second experiment, the sorption isotherm of a mix prepared following exactly the above described TTI preparation procedure with a sample of BLA solution (dilution in Buffer bis tris pH 6.9, ultra centrifugation...) was determined (figure annex II 2). It appears that moisture adsorption starts to increase significantly around a water activity of 0.5 (50% equilibrium relative humidity). This observation is of importance since it explains that adsorption of moisture from the air toward the mix can occur because of the % relative equilibrium humidity of the air inside a laboratory (45% to 55%).

On the basis of this information it became important to analyze the kinetics of moisture adsorption on the mix after drying for one hour in an oven and the kinetics of desorption of water from the mix when placed inside an oven at a temperature well above room temperature. Therefore, a third experiment was run, which consisted in submitting 6 pans filled with freshly freeze-dried mix to heating in an oven followed by storage during 24 hours inside a dessicator containing P_2O_5. Each pans and its content had been weighed before to be put inside the dessicator and no variation of weight was observed during the storage in the dessicator. Three pans were exposed to 102°C during 5, 10 and 60 min respectively. Three other pans were exposed to 60°C during 60, 120 and 180 min respectively. At each time, each pan was weighed immediately and one of them was kept for residual activity reading while the others were immediately placed back inside

the oven. Table annex II.1 provides data about water desorption from a freeze-dried mix stored inside an oven as a function of exposure time and temperature. Table annex II.2 shows water adsorption on a freeze-dried mix stored at room temperature in function of time. From table annex II.1 it can be concluded that a mix prepared with a BLA solution, freeze dried and stored in a dessicator during 24 hours still contains water and is totally dried after 60 min at 60°C or after 10 min at 102°C. No activity decay was observed whatever the case. From table annex II..2 it can be concluded that the mix rapidly adsorbs moisture and that an equilibrium with an atmosphere at 22°C and 55% relative humidity is achieved after about 15 min. The observed average gain of water is only 0.23% with an important standard deviation of 0.05% that represents 22% of the average gain of water. It comes down to say that 50 mg of freeze dried mix stored 24 hours in a dessicator would adsorb 0.12 mg ± 0.02 mg of water. Due to the fact that 50 mg of mix prepared with BLA according to the procedure described above contains no more than 0.07 mg of enzyme, it is obvious that the relatively high standard deviation in water adsorption can lead to important differences in the mass ratio "enzyme/water content" from one pan to one another, that can, in turn, lead to important differences in terms of thermal inactivation and finally in terms of process-value calculated with different TTIs (capsules) from a same TTI preparation batch.

An illustration of this is shown with Figure annex II.3. This figure corresponds to a validation under non-isothermal conditions (see part 3.3 and 3.4) of a TTI system (called BLA0.48) made of encapsulated mix (made with *Bacillus licheniformis* α-amylase) equilibrated at a relative equilibrium humidity of 48% at 41°C by use of hermetically sealed containers containing saturated salt solutions of Magnesium Nitrate ($MgNO_3$) according to Greenspan tables (1977). It appeared that for similar heat treatments, that is for similar or almost similar actual process-values Ft-T derived from equation 1.8, an important variation of the Process-value FTTI derived from the response of the TTI (equation 1.11) could be observed and that this variation was increasing with the actual process-value. Whatever the kinetic parameters used, derived from isothermal data or non isothermal data, a non negligible % error around 20% in the prediction of the actual process value (see equation 8) was consequently observed. As shown by figure 3.4 this % error of prediction was seriously decreased up to around 13% when plotting averaged FTTI versus the averaged corresponding Ft-T. Nethertheless, the important variations observed on FTTI derived from several TTI submitted to similar heat treatments is undoubtedly due to non negligible variations from pan to pan of the amount of water taken up by the mix inducing various ratio "enzyme/water content" from one pan to one another. This is probably not the only reason. A second reason could also be hysteresis phenomena. It has indeed been shown above that an equilibrium between a mix and the %RH of the air, which is often between 45% and 55%, could be achieved in few minutes. In the case of BLA48, the equilibrium relative humidity was around 51% when filling the pans with freeze-dried mix. The consequence is that, during the filling of the pans, partial equilibration with air humidity induces an excess of adsorbed water inside the pans.

From one pan to another the partial equilibration time is not exactly the same (the first filled pans are submitted during a longer time to the humidity of the air than the last ones). This induces heterogeneities in water content of the pans before they are placed under 48% humidity atmosphere. Few pans are below and others are above the expected equilibrium relative humidity that is 48%. Depending on the case, water adsorption or water desorption occurs under 48%ERH exposure, inside the hermetically closed containers with saturated Magnesium nitrate solution at 41°C. But at the equilibrium under 48% ERH the pans where desorption occurred have a higher water content (mass %) than pans where adsorption occur. This comes down to say that the desorption isotherm curve can not be perfectly superposed to the adsorption isotherm and this phenomena is called "hysteresis" (Fennema, 1985).

To overcome any hysteresis we decided to investigate only TTI systems based on a mix strongly dehydrated by oven heating at a temperature well above the ambiant and encapsulation by sealing within few seconds after heating.

EXAMPLE III

Experimental evidence for the necessity to coat capsules with silicone (step 18).

This step appeared to be necessary to overcome entrance of water inside the capsules after sealing and subsequent drastic changes in the response of the TTI. Indeed, according to our experiments, the DSC pans are not totally hermetically sealed, even when respecting the sealing rules from Perkin-Elmer. To show it, a small plastic net containing TTIs was placed in contact with the tip of a thermocouple placed, with help of a specially designed frame (see figure annex III.1), at the center of a can filled with water. The frame was made of three teflon rings fixed on four thin stainless steel rods. The two rings at the bottom and top were fixed while the middle ring could be adjusted up and down the length of the steel rods to a desired position. The net containing the TTIs was held in place by two thin non-elastic strings traversing across the diameter of the middle ring and tied to the middle ring on either side. The net was attached to the two strings using small nodes. Figure 3.5 describes a can equipped with a thermocouple and a net containing TTIs. Once equipped, the can was submitted to a static heating process in a single basket (height 370mm, depth 700mm and width 400mm) Steriflow simulator (microflow type 911R n°877, Barriquand, France). Heating medium temperatures were recorded every 15 seconds using calibrated copper-constantan thermocouples (Ellab, Denmark) by a CMC-92 data acquisition system (TR9216, Ellab, Denmark).The actual process value was calculated on-line (with Tref=121.1°C and z= zTTI based on equation 1) by using the software Pcsoft92 (Ellab, Denmark) and a recording step of 15 seconds. A first run leading to a process value of 15.54 min with 4 capsules non protected by silicone was performed. A second run leading to a process-value of 14.85 min was performed with 1 capsule over-sealed (thickness=2.7 mm) without silicone, 1 capsule strongly over sealed (thickness=2.65 mm) without silicone and 3 capsules normally sealed and coated with silicone. Table 3.3 introduces the process values FTTI (equation 1.11) achieved with the TTIs depending on their level of protection. From table 3.3, it appears clearly that a large overestimation of the true process-value is achieved when using normally sealed and over-sealed capsules not coated with silicone. This is due to some water entrance inside the capsules after sealing and especially during the heating-process. This entrance of moisture decreases drastically the D-value of the TTI during heating thus leading to residual activities much smaller than those observed avoiding water penetration. Over-sealing, however, appears as decreasing the entrance of water inside the capsule. It is interesting to mention, here, that in spite of the moisture entrance inside the capsules, the mix appeared visually as a totally dried powder when opening the capsules after heat treatment. Due to the fact that previous experiments showed that 0.05 mg of moisture uptake was enough to make the mix (of one pan) sticky we could conclude that the amount of water to penetrate inside the pan during the heating process is smaller than 0.05mg. This experiment shows the importance of the moisture control at all levels of preparation and use of the TTI.

EXAMPLE IV

Method to calculate a correction factor to apply to the Process- value deduced from a single component TTI showing a different z-value than the one of the target attribute under interest.

This section introduces computer calculations based on a one-dimensional explicit forward finite difference heat transfer model (Silva and others, 1992). The computer calculations are based on the fact that, for a conductive heating infinite cylinder at uniform initial temperature and with a constant surface temperature, the heating rate parameter f_h, defined as the time required to change the difference between heating medium and product temperature by a factor of 10, can be expressed as a function of the thermal diffusivity α and the radius of the infinite cylinder R (equation annex IV.1) (Ball and Olson, 1957). Hence, based on equation annex IV.1, it is possible to define infinite cylinders simulating foods with known f_h-values.

For instance, we performed a theoretical study where 21 various theoretical food products with given f_h-values in the range 0-150 min and 15 various target process values in the range 3 to 80 min (with z=10°C and T_{ref}=121.1°C) at the center of these products were considered. 16 different holding temperatures for the theoretical process were chosen between 110 and 140°C (every 2°C). Taking all the different values of f_h, holding temperatures and target process values, it was possible, with a z=10°C, an initial temperature of the products equal to 20°C and a temperature of the cooling medium equal to 20°C (Rq: it would be possible to repeat the same experiment with other parameters), to generate (21*15*16)=5040 corresponding time temperature profiles with, for each of them, a corresponding holding time at the holding temperature (rq: in our experiment the come up time (CUT), that is the time required for the heating medium to reach the holding temperature was considered as zero. In case the process under consideration shows CUT different from 0, this CUT can be transformed in an equivalent time at the holding temperature of the process and added to the holding time of the process.). Table annex IV.1 shows the processing conditions used to generate conduction heating profiles with a finite difference heat transfer model.

The following step consisted in integrating these 5040 time-temperature profiles (each corresponding to a combination "target process value, holding temperature, f_h, z=10°C) with 9 different z-values from 5.5° to 16.5° by 1°C, that could represent the z-value of a TTI different from 10°C (z-value=10°C is currently used as a reference for the calculation of process-values with spores of *Clostridium botulinum* as a target). A Simpson integration routine (Carnahan and others 1969) was used for the integration. Hence, for each of the above created 5040 time temperature profiles, 9 process-values achieved with a z-value different from 10° C

were obtained. Each of the 9 process-values achieved with a z-value different from 10°C was compared to the process value achieved with z=10°C, which was, of course, equal to the target process-value under consideration. Hence, for each combination "f_h, holding temperature, holding time, and z target", a % correction, called C, to apply to the process value derived from the TTI with a z different from 10°C could be calculated with equation annex IV.2: In this way 5040*9=45360 correction factors C could be calculated.

$$f_h = \frac{0.393 \cdot R^2}{\alpha} \qquad \text{(Annex IV.1))}$$

$$\%\text{correction} = C = -\left(\frac{{}^{zTTI}F_{121.1°C} - {}^{10°C}F_{121.1°C}}{{}^{zTTI}F_{121.1°C}}\right) \cdot 100 \qquad \text{(Annex IV.2)}$$

Finally, by collecting, for each given "f_h, zTTI different from 10°C" combination, the corresponding holding temperatures, holding times and % correction C, it was possible to obtain 240 points in a 3 dimensional representation where the correction factor C are plotted versus all combinations of "holding time (ht) and holding temperature (T)". The following step consisted to perform a modeling, by nonlinear regression (Sas, 1982), of the 21*9=189-three-dimensional graphs corresponding to the 189 clouds of 240 points. A good fitting (small residues) could be achieved in the 189 cases using an equation of the following form where 11 parameters had to be found for each "f_h, z_{TTI}" combination.

$$C=a_0+a_1*T+a_2*ht+a_3*ht*T+a_4*(ht/T)+a_5*(1/ht)+a_6*(1/T)+a_7*(1/(ht*T))+a_8*(1/(ht*T)^2)+a_9*(1/(T^2))+a_{10}*(1/(ht^2)) \qquad \text{(Annex IV.3)}$$

with: T=holding temperature (110°C to 140°C by 2°C).

ht=holding time (hours).

C=% correction to apply to the process value derived from a single component TTI showing a z value different from 10°C. The correction has to be applied as follows: ${}^{10°C}F_{121.1°C}={}^{zTTI}F_{121.1°C}+({}^{C*\,zTTI}F_{121.1°C}/100)$.

Once the 189 equations were obtained, the corresponding graphs were plotted with help of SAS software (SAS 1982).

For instance, figure annex IV.1 shows an example for a single component TTI with a z=13.5°C used in a product with a f_h=4.379 min. Figure annex IV.2 shows a second example of a single component TTI with a z=16.5°C used in a product with a f_h=4.379 min. Of course, only a part of these figures correspond to realistic conditions met in the industry.

EXAMPLE V

Possibility to use multi-component TTIs to determine the process-value inside a product when no single-component TTI showing the z-value of the target attribute under interest is available.

-this method consists of using the already known multi-component TTI concept, mentioned above.

When using two-component TTIs, this method is based on the hypothesis that for relative small differences between the z -value of the target attribute of interest and the z-value of the TTI, the difference in the F values for the target attribute of interest and the TTI, ΔF, is proportional to the correspondent difference of the z values, Δz. Taking into account that $\Delta F=0$ when $\Delta z=0$, we have:

$$\Delta F=\alpha^*\Delta z \qquad \text{(annex V.1)}$$

The parameter α in the above equation can be determined from two F-values (F_1 and F_2) calculated from the responses of two TTIs (or from a double response TTI system) each one of them characterized by different z-value (z_1 and z_2) as $\alpha=(F_2-F_1)/(z_2-z_1)$. Having thus calculated the parameter α, for a given thermal treatment, then by using the F-value associated with the one z-value of the TTI and the previous equation, one can calculate the F-value for a target attribute with a particular z target. If the z-values of the two components of the TTI are above or below the one of the target attribute under interest, then the actual process-value can be evaluated by extrapolation. If the z-values of the two components of the TTI are above and below the one of the target attribute under interest, then the actual process-value can be evaluated by interpolation.

When using triple-component TTIs, a simple non linear regression can be performed with F_1, z_1, F_2, z_2, F_3, z_3 to find the parameters a, b and c of the following equation:

$$F=a^*z^2+b^*z+c \qquad \text{(annex V.2)}$$

Then, by applying the target z-value to this equation, it becomes possible to evaluate the actual F-value corresponding to this target attribute.

In order to evaluate the potentials of the multi-component TTIs to provide a good estimation of the actual process value corresponding to a target attribute with a z=10°C, we integrated the 5040

time-temperature profiles described in annex IV with a z-value of 10°C to obtain the actual process-value corresponding to this target attribute, $^{z=10°C}F_{121.1°C}$. In a second step, we integrated the 5040 profiles with z=8.5°C, z=13.5°C and z=16.5°C that could be the z-values of the two or three components of a multi-component TTIs, and collected the corresponding process-values $^{z=8.5°C}F_{121.1°C}$, $^{z=13.5°C}F_{121.1°C}$ and $^{z=16.5°C}F_{121.1°C}$. In a third step, the following cases were taken as examples of multi-component TTIs:

Case 1: Two-component TTI with z=13.5°C and z=16.5°C.
Case 2: Two-component TTI with z=8.5°C and z=13.5°C.
Case 3: Three-component TTI with z=8.5°C, z=13.5°C or z=16.5°C.

In case 1, the evaluation of the actual $^{z=10°C}F_{121.1°C}$ was performed by linear extrapolation
In case 2, the evaluation of the actual $^{z=10°C}F_{121.1°C}$ was performed by linear interpolation.
In case 3, the evaluation of the actual $^{z=10°C}F_{121.1°C}$ was performed by non linear interpolation.
In allcases the responses of the multi-component TTI, obtained by extrapolation or interpolation, were called F(multiTTI).

For each of the three examples of multi-component TTIs, a % correction C was calculated using equation annex V.3:

$$\%\text{correction} = C = -\left(\frac{F(\text{multiTTI}) - \text{actual}^{10°C}F_{121.1°C}}{F(\text{multiTTI})}\right)\cdot 100 \qquad \text{(annex V.3))}$$

Where C=% correction to apply to the process value F(multiTTI) derived from a multi-component TTI. The correction has to be applied as follows: $^{10°C}F_{121.1°C}$=F(multiTTI)+(C*F(multiTTI)/100).

Finally, for the 21 f_h mentioned in annex IV, it was possible, for each of the three multi component TTIs, to plot the 240 % correction C-values versus the 240 corresponding combinations "holding temperature (T), Ftarget". Table annex V.1 provides details about the potential of the three multi-component TTIs described above to evaluate properly the actual process value $^{z=10°C}F_{121.1°C}$, for products with f_h =10.81 min, f_h=20.11 min and f_h=75.16 min. Table annex V.2 allows to compare the performances of the above described multi-component TTIs with the ones of single-component TTIs showing similar z-values.

Several observations can be made:

1-On the basis of the performances described in tables annex V.1 and annex V.2, it appears clearly that, in all cases, the multi-component TTIs allow to determine the actual process value with a significantly better accuracy than the single component TTIs.

2-whatever the f_h, the three-component TTI is logically more accurate than the two component TTIs. However, the two-component TTI with z=8.5°C and z=13.5°C provides a level of accuracy almost equivalent to the one of the three component TTI. The two component-TTI with z=13.5°C and z=16.5°C also shows an interesting level of accuracy but it appears that, according to the highest negative % correction C and highest positive % correction C observed, its accuracy may decrease strongly in a given range of "Ftarget,T" combinations.
In order illustrate the variation of accuracy of the above described multi-component TTIs, non-linear regressions were performed on (annex V.4) where 11 parameters (a_0 to a_{10}) had to be found for each "fh, multiTTI" combination. 21*3=63 non-linear regressions were performed on 63 datasets of 240 points.

$$C=a_0+a_1*T+a_2*Ftarget+a_3*Ftarget*T+a_4*(Ftarget/T)+a_5*(1/Ftarget)+a_6*(1/T)+a_7*(1/(Ftarget*T))+a_8*(1/(Ftarget*T)^2)+a_9*(1/(T^2))+a_{10}*(1/(Ftarget^2)) \quad \text{(annex V.4)}$$

with: T=holding temperature (110°C to 140°C by 2°C)

Ftarget=actual ${}^{10°C}F_{121.1°C}$ achieved by integrating the time-temperature profiles with z=10°C.

C=% correction to apply to the process value derived from a single component TTI showing a z value different from 10°C. The correction has to be applied as follows:

Ftarget=actual ${}^{10°C}F_{121.1°C}$=F(multiTTI)+(C*F(multiTTI)/100).

Figures annex V.1, annex V.2 and annex V.3 show the %correction C as a function of Ftarget and holding temperatures T for the two-component TTI with z=13.5°C and z=16.5°C. These 3 graphs show clearly the conditions in which the use of such two-component TTI could be suitable and the conditions in which a low level of accuracy on the evaluation of the actual process-value ${}^{10°C}F_{121.1°C}$ may be achieved without use of a % correction deduced from equation annex V.4.

The most important observation here, is that, in a broad and realistic (with regard to the thermal processing of foods) range of target process-values Ftarget, holding temperatures T and f_h, the three-component TTI with z=8.5°C 13.5°C and 16.5°C and the two component TTI with z=8.5°C and 13.5°C, show, in all cases, a good level of accuracy in process value determination. Hence, without applying any correction and without taking into account the thermal characteristics of the product nor the thermal characteristics of the process, these multi-component TTIs may guarantee a high level of accuracy in terms of process value determination.

Conclusion:

On the basis of the above results, it appears clearly that multi-component TTIs with z-values close enough to the target attribute z-value, may be used as an extremely efficient approach in case of the lack of a single component TTI with a z-value equal to the one of the target attribute (in our example 10°C). They allow the determination of the process-values with a good accuracy without any necessity to know the thermal properties of the food under concern nor the thermal characteristics of the process. Moreover, if, on one hand, the use of multi-component TTIs may necessitate more time to be used due to the several readings required, on the other hand, their enormous advantage is that they could allow the determination of thermal impacts on target attributes with various z-values (not necessarily 10°C). This last point may largely counterbalance the necessity of several readings (at least two) inherent to the use of multi-component TTIs. From a practical point of view, the above mentioned results show clearly that the finding of an enzymic TTI with a z-value close to 8.5°C, prepared following the above described TTI procedure and based on another substrate specificity than the *Bacillus licheniformis* alpha-amylase mentioned above, may allow to prepare an efficient and polyvalent two-component TTI, based on a mix containing this enzyme and *Bacillus licheniformis* alpha-amylase.

EXAMPLE VI

Application of the above described TTI preparation procedure to purified cucumber Pectine-Methyl Esterase (PME): possibility to prepare TTIs for pasteurisation

When applying the above described TTI preparation procedure to a purified cucumber PME, the following inactivation curve is obtained at 102°C (figure annex VI.1). The D-value observed at 102°C was 65.8 min. Although only one temperature exposure was investigated (no z-value determined), this result shows that (i) it is possible to strongly increase the thermal stability of a plant related enzyme such as cucumber PME up to temperatures higher than 100°C (ii) hence, it appears possible to use the above described TTI preparation method also to prepare TTIs useful for high pasteurisation processes (90-110°C).

REFERENCES

Adams, J.B. and Langley, F.M. (1998). Nitrophenyl glucoside hydrolysis as a potential time-temperature integrator reaction. Food Chem., 62(1), 65-68.

Arrhenius S. (1889). Über die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. Zeitschrift für Physikalische Chemie, 4(2), 226-248.

Ball and Olson. Sterilization in food technology (Chapters 5 and 6).1957.

Bean, P., Dallyn, H. and Ranjith, H. (1979). The use of alginate spore beads in the investigation of ultra-high temperature processing. J. Food Technol., 281-294.

Berry, M.R., Singh, R.K. and Nelson, P.E. (1989). Kinetics of methylmethionine sulfonium in buffer solutions for estimating thermal treatment of liquids foods. J. Food Proc. Preserv., 13, 475-488.

Bigelow, W.D. (1921). The logarithmic nature of thermal death time curves. J. Infect. Dis., 29(5), 528-536.

Brown, K.L, Ayres, C.A., Gaze, J.E. and Newman, M.E. (1984). Thermal destruction of bacterial spores immobilized in food/alginate particles. Food Microbiol., 1, 187-198.

Danielson, E. (1982). Sterilization process indicators: biological versus chemical. Medical instrumentation, 16(1), 52.

Burton, H., Perkin, A.G., Davies, F.L. and Underwood, H.M. (1977). Thermal death kinetics of Bacillus stearothermophilus spores at ultra high temperatures. III. Relationship between data from capillary tube experiments and from UHT sterilizers. J. Food Technol., 12, 149-161.

De Cordt, S., Hendrickx, M., Maesmans, G. and Tobback, P. (1992a). Immobilized α-amylase from Bacillus licheniformis: a potential enzymic time-temperature integrator for thermal processing. Int. J. Food Sci. Technol., 27, 661-673.

De Cordt, S., K. Vanhoof, J. Hu, G. Maesmans, M. Hendrickx, and Tobback,P. (1992b). Thermostability of soluble and immobilized alfa-amylase from Bacillus licheniformis. Biotechnology and Bioengineering, 40, 396-402.

De Cordt, S., Hendrickx, M., Maesmans, G. and Tobback, P. (1993). The influence of polyalcohols and carbohydrates on the thermostability of α-amylase. Biotechnology and Bioengineering, 43(2), 107-114.

De Cordt, S., Avila, I., Hendrickx, M. and Tobback, P. (1994). DSC and protein-based time-temperature integrators: case study of α-amylase stabilized by polyols and/or sugar. Biotechnology and Bioengineering, 44(7), 859-865.

Favetto, G.J., Chirife, J., Scorza, O.C. and Hermida, C. (1988). Color-changing indicator to monitor the time-temperature history during cooking of meats. J. Food Protect., 51(7), 542-546.

Favetto, G.J., Chirife, J., Scorza, O.C. and Hermida, C. (1989). Time-temperature integrating indicator for monitoring the cooking process of packaged meats in the temperature range of 85-100°C. United States Patent, 4 834 017

Gaze, J.E., Spence, L.E., Brown, G.D. and Holdsworth, S.D. (1990) 'Microbiological assessment of process lethality using food/alginate particles' in Technical memorandum n°580, Campden Chorleywood Food Research Association, 1-47

Guiavarc'h, Y., Deli, V., Van Loey, A. and Hendrickx, M. (2002). Development of an enzymic time temperature integrator for Sterilization processes based on *Bacillus licheniformis* α-amylase at reduced water content. Accepted for publication in J. Food Sci.

Hayakawa K.I. (1970) experimental formulas for accurate estimation of transient temperature of food and their application to thermal process evaluation. Food Technol., 24,1407.

Haentjens T. H, Van Loey A. M., Hendrickx M.E. and Tobback P.P. (1998).The Use of alpha-amylase at Reduced Water Content to Develop Time Temperature Integrators for Sterilization Processes.. Lebensmittel-Wissenschaft und Technologie, 467-472.

Hendrickx, M., Maesmans, G., De Cordt, S.,Noronha, J., Van Loey, A., Willockx, F and Tobback, P. (1993). Advances in process modelling and assessment: the physical mathematical approach and product history integrators. In Minimal Processing of Foods and Process Optimization (Singh, R.P. and Oliveira, F.A.R., eds), pp. 315-335; CRC press.

Hendrickx M., Maesmans G., De Cordt S., Noronha J., Van Loey A. and. Tobback P. (1995). Evaluation of the integrated Time-Temperature Effect in thermal processing of foods.. Critical reviews in food sciences and nutrition, 35(3) : 231-262.

Heppel, N.J. (1985). Measurement of the liquid-solid heat transfer coefficient during continuous sterilization of foodstuffs containing particles. In Proceedings of IUFOST symposium on aseptic processing and packaging of foods, september 9-12, Tylosand, Sweden, 108-114.

Hersom, A.C. and Shore, D.T. (1981). Aseptic processing of foods comprising sauce and solids. Food Technol., 35(4), 53-62.

Hunter, G.M. (1972). Continuous sterilization of liquid media containing suspended particles. Food Technol. Aust., April, 158-165.

Jason, A.C. (1983). A deterministic model for monophasic growth of batch cultures of bacteria. J. Microbiol. Serol., 49, 513-536.

Jones, A. and Pflug, I. (1981). Bacillus coagulans, FRR B666, as a potentional biological indicator organism. J. Parenter. Sci. Technol., 53(3), 82-87.

Kim, H.-J. and Taub, I.A. (1993). Intrinsic chemical markers for aseptic processing of particulate foods. Food Technol., 47(1), 91-99

Lund D.B. (1977). Design of thermal processes for maximizing nutrient retention. Food Technol., 31 (2), 71-78.

Maesmans, G., Hendrickx, M., Weng, Z., Keteleer, A. and Tobback, P. (1990). Endpoint definition, determination and evaluation of thermal processes in food preservation. Belgian J. Food Chem. Biotechnol., 45(5), 179-192.

Maesmans G., Hendrickx M., De Cordt S., Van Loey A., Noronha J.and Tobback P. (1994). Combined use of the equivalent point method and a multi-component time-temperature integrator in thermal process evaluation : influence of kinetic characteristics and reference temperature.. Food Control, Volume 5, No 4, 249-256.

Maesmans G., Hendrickx M., De Cordt S. and Tobback P. (1993). Theoretical considerations on design of multicomponent time-temperature integrators in evaluation of thermal processes. Journal of Food processing and preservation, volume 17, 369-389

Matthiasson, E. and Gudjonsson, T. (1991). Enzymes as thermal-time indicators in food, ITI, Reykjavik.

Mulley, A., Stumbo, C. and Hunting, W. (1975a). Kinetics of thiamine degradation by heat. A new method for studying reaction rates in model systems and food products at high temperatures. J. Food Sci., 40(5), 989-992.

Mulley, A., Stumbo, C. and Hunting, W. (1975b). Kinetics of thiamine degradation by heat. Effect of pH and form of the vitamin on its rate of destruction. J. Food Sci., 40(5), 993-996.

Ocio, M.J., Salvador-Fernandez, P., Rodrigo, M., Periago, P. and Martinez, A. (1997). A time temperature integrator for particulated foods: thermal process evaluation. Zeitschrift-fuer-Lebensmittel-Untersuchung-und-Forshung, 205(4), 325-328.

Orta-Ramirez, A., Merill, J.E.,Smith, D.M. (2000). PH affects the thermal inactivation parameters of R-phycoerythrin from *Porphyra yezoensis*. J. Food Sci., 65(6), 1046-1050.

Pflug, I.J. and Smith, G.M. (1977). The use of biological indicator for monitoring wet heat sterilization processes. Sterilization of medical products (Gaughran, E.R.L. and Kereluk, K., eds), pp. 193-230, Johnson and Johnson, New Brunswick, N.J.

Pflug I.J. and Christensen R. Converting an F-value determined on the basis of one z-value to an F-value determined on the basis of a second z-value.. Journal of food Science. Vol. 45, 35-40 (1980).

Pflug, I.J., Jones, A. and Blanchett, R. (1980a). Performance of bacterial spores in a carrier system in measuring the F0-value delivered to cans of food heated in a Steritort. J. Food Sci., 45(4), 940-945.

Pflug, I.J., Smith, G., Holcomb, R. and Blanchett, R. (1980b). Measuring sterilizing values in containers of food using thermocouples and biological indicator units. J. Food Protect., 43(2), 119-123.

Pflug, I.J. and Odlaug, T.E. (1986). Biological indicators in the pharmaceutical and medical device industry. J. Parenter Sci. technol., 40(5), 242-248.

Pflug I.J. (1987a). Textbook for an introduction course in the microbiology and engineering of sterilization processes. Sixth edition, environmental Sterilization Laboratory, Minneapolis, USA.

Pflug I.J. (1987b). Endpoint of a preservation process. J. Food Prot., 50(4), 347-351.

Pflug I.J. (1987c). Factors important in determining the heat process value F for low acid canned foods. J. Food Prot., 50(6), 528-533.

Rodriguez, A.C. and Teixeira, A.A. (1988). Heat transfer in hollow cylindrical rods used as bioindicator units for thermal process validation. Trans. ASAE, 31(4), 1233-1236.

Rönner, U. (1990a). A new biological indicator for aseptic sterilization. Food Technology International Europe, 90, 43-46.

Rönner, U. (1990b). Bioindicator for control of sterility. Food Laboratory News, 22(6:4), 51-54.

Sadeghi F. and Swartzel K.R. (1990), Time Temperature equivalence of discrete particules during thermal processing. J. Food Sci., 55 (6), 1696-1698, 1739.

SAS Institute, Inc. 1982. SAS User's guide: Statistics. 4nd ed. Cary, North Carolina: Statistical Analysis System Institute. 1686p.

Sastry, S.K., Li, S.F., Patel, P., Konanayakam, M., Bafna, P., Doores, S. and Beelman, R.B. (1988). A bioindicator for verification of thermal processes for particulate foods. J. Food Sci., 53(3), 1528-1531.

Segner, W.P., Ragusa, T.J., Marcus, C.L. and Soutter, E.A. (1989). Biological evaluation of a heat transfer simulation for sterilizing low-acid large particulate foods for aseptic packaging. J. Food Proc. Preserv., 13, 257-274.

Silva, C., Hendrickx, M., Oliveira, F. and Tobback, P. (1992). Optimal sterilization temperatures for conduction heated foods with finite surface heat transfer coefficients. Journal of Food Science, 57(3), 743-748.

Smout C., Van Loey A. and Hendrickx M. (1998). Heat Distribution in Industrial-scale Water Cascading (Rotary) Retort. Journal of Food Science. Volume 63, no 5, 882-886.

Smith, D.M. (1995). Immunoassays in process control and speciation of meats. Food Technol., 49(2), 116-119.

Stoforos N.G. and Taoukis P.S. (1998).A theoretical procedure for using multiple responses time-temperature integrators for the design and evaluation of thermal processes. Food control, volume 9, no 5, 279-287.

Stoforos N.G., Noronha J., Hendrickx M and Tobback P. (1997). A critical analysis of mathematical procedures for the evaluation and design of in-container thermal processes for food. CRC Critical Reviews in Food Science and Nutrition. 37(5): 411-441.

Swartzel, K.R. (1986). Equivalent point method for thermal evaluation of continuous flow systems. J. Agric. Food Chem.,34, 396-401.

Swartzel K.R, Ganesan S.G., Kuehn R.T., Hamaker R.W. and Sadeghi F. (1991). Thermal memory cell and thermal system evaluation . US patent 5.021 981.

Taoukis P.S. and Labuza TP (1989a). Applicability of time temperature indicators as shelf life monitors of food products. J. Food Sci., 54 (4). 783-788.

Taoukis P.S. and Labuza T.P. (1989b). Reliability of time temperature indicators as food quality monitors under nonisothermal conditions. J. Food Sci., 54 (4), 789-791.

Tsou C.L. (1986), Location of the active sites of some enzymes in limited and flexible molecular regions. TIBS, October, 427-429.

Van Loey A., Ludikhuyze L., Hendrickx M., De Cordt S.and Tobback P. (1995a). Theoretical consideration on the influence of a z-value of a single component Time/Temperature Integrator on thermal process Impact Evaluation Journal of food protection, vol 58, No. 1, page 39-48.

Van Loey A., Hendrickx M., Ludikhuyze L., Weemaes C., Haentjens T., De Cordt S. and Tobback P. Potential *Bacillus subtilis* α-amylase based Time Temperature Integrators to evaluate pasteurization processes. (1995b). Journal of food protection, vol.59, No.3, pages 261-267.

Van Loey A., Hendrickx M., De Cordt S., Haentjens T. and Tobback P. (1996). Quantitative evaluation of thermal processes using time-temperature-integrators. Trends in Food Science and Technology 7 (1) : 16-26.

Van Loey A., Arthawan A., Hendrickx M., Haentjens T. and Tobback P. (1997a).The development and use of an α-amylase based Time Temperature Integrator to evaluate in-pack pasteurisation processes. Lebensmittel-Wissenschaft und Technologie, 30 (1). 94-100.

Van Loey A.M., Haentjens T.H., Smout C., Hendrickx M.E. and Tobback P.P. (1997b), The development of an enzymic Time-Temperature-Integrator to monitor thermal efficacy of sterilisation of low acid canned foods. Food Biotechnology, 11 (2), 169-188.

Van Loey A., Fransis A., Hendrickx M., Maesman G., Tobback P. (1994a) Kinetics of thermal softening of white beans evaluated by sensory panel and the FMC Tenderometer. Journal of Food Processing and Preservation, 18, 407-420.

Van Loey A., Fransis A., Hendrickx M., Maesman G., Tobback P (1994b).optimizing thermal process for canned white beans in water cascading retorts. Journal of Food Science, 59(4),828-832.

Van Loey A., Noronha J., Hendrickx M and Tobback P. (1995c). The optimization of product quality during thermal processing : a case-study on white beans in brine. In "Minimal processing of Foods and Process Optimisation", ed. F. Oliveira and P. Singh, CRC press, chapter 25, 369-388.

Weemaes C., De Cordt S., Goosens K., Ludikhuyze L., Hendrickx M., Heremans K., Tobback P. (1996).High pressure, thermal and combined Pressure-Temperature stabilities of alpha amylase from Bacillus species. Biotech. Bioeng., 50, 49-58.

Wen Chin, L. (1977). Disaccharide hydrolysis as a predictive measurement for the efficacy of heat sterilization in canned foods. Ph. D. thesis, Departement of Food Science and Nutrition, University of Massachusetts, Amhers, Massachusetts, USA.

Weng Z., Hendrickx M., Maesmans G. and Tobback P. (1991a). Immobilized peroxidase : a potential bioindicator for evaluation of thermal processes. J. Food. Sci., 56(2), 567-570.

Weng Z., Hendrickx M.,Maesmans G., Gebruers K. and Tobback P. (1991b). Immobilized peroxidase : Thermostability of soluble and immobilized horseradish peroxidase. J. Food. Sci., 56(2), 574-578.

Witonsky, R.J. (1977). A new tool for the validation of the sterilization of parenterals. Bull. Parenter. Drug Assoc., 11(6), 274-281.

Wunderlich, J. (1995). Temperature bio-indicators based on immobilized enzyme for the controlling of continuous HTST heating of particulate foods and other heating processing. Proceedings of New Shelf-life Technolgies and safety assessments, VTT symposium, Helsinki, Finland, (Ahvenainen, R., Mattila-Sandholm, T. and Ohlsson, T., eds), pp. 197.

Yawger, E.S. (1978). Bacteriological evaluation for thermal process design. Food Technol., 32(6), 59-62.

Yih-Chih-Hsu-I. (1997). Identification and verification of an endogenous time-temperature integrator to determine processing adequacy of roast beef. Thesis in Dissertation Abstracts International,-B, 58(5) 2196, order n° DA9734135, 148 pp.

LIST OF TABLES

Box 1 Commonly used terminologies to describe heat inactivation kinetics

Thermal Death Time concept (i.e. first order reaction)	Arrhenius concept
-Commonly used in	
thermobacteriology and thermal processing	chemical reaction kinetics
-A measure for the rate of reaction	
Decimal reduction time D (min) Defined as the time at constant Temperature required to reduce the initial response value by 90%	**reaction rate constant k** ((response units)$^{1-n}$ (time units)$^{-1}$)
$D = \frac{\ln 10}{k}$	
-Temperature coefficient	
z-value (°C) defined as the temperature increase necessary to obtain a tenfold decrease of the D-value	**activation energy E_a (J/mol)**
$D = D_{ref} \cdot 10^{\left(\frac{Tref - T}{z}\right)}$	$k = k_{ref} \cdot \exp\left(\frac{E_a}{R_g}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right)$

Table 2.1 : State of the art of TTIs (Van Loey, 1996)

Origin	Application	Principle	Activation energy or z-value	Reference
Microbiological				
extrinsic	dispersed	*Bacillus stearothermophilus*	NR[1]	Burton *et al.* (1977)
		Bacillus coagulans	NR	Jones & Pflug (1981)
		Bacillus subtilis	NR	Pflug & Odlaug (1986)
		Clostridium sporogenes	NR	Pflug & Odlaug (1986)
		Inoculated Pack[a]		Yawger (1979)
	Permeable	*Bacillus stearothermophilus* in alginate	8.5°C	Bean *et al.* (1979)
		Bacillus stearothermophilus in alginate	11.4-11.8°C	Brown *et al.* (1984)
		Clostridium sporogenes in alginate	12.5-12.7°C	Brown *et al.* (1984)
		Bacillus stearothermophilus in alginate	9°C	Heppel (1985)
		Bacillus stearothermophilus in alginate	NR	Sastry *et al.* (1988)
		Clostridium sporogenes in a turkey cube	8.5°C	Segner *et al.* (1989)
		Bacillus stearothermophilus in polyacrylamide gel	11.7°C	Rönner (1990a, b)
		Bacillus stearothermophilus in alginate	8.8-9.1°C	Gaze *et al.* (1990)
	Isolated	*Bacillus anthracis* in perspex	60°C	Hunter (1972)
		Bacillus stearothermophilus in plastic	7.8-10°C	Pflug *et al.* (1980a, b)
		Bacillus stearothermophilus in glass bulb	10°C	Hersom & Shore (1981)
		Bacillus stearothermophilus in aluminium	NR	Rodriguez & Teixeira (1988)
Enzymic				
intrinsic	dispersed	enzyme-linked immunosorbent assay for lactate dehydrogenase	NR	Smith (1995)
extrinsic	Permeable	β-galactosidase in alginate	NR	Matthiasson & Gudjonsson (1991)
		lipase in alginate	NR	Matthiasson & Gudjonsson (1991)
		nitrate reductase in alginate	NR	Matthiasson & Gudjonsson (1991)
		immobilized amylase in alginate matrix	NR	Wunderlich (1995)
	isolated	immobilized peroxidase in decanol	11.6°C	Weng *et al.* (1991)
		immobilized peroxidase in dodecane	10.1°C	Weng *et al.* (1991)
		immobilized *Bacillus licheniformis* α-amylase	302kJ/mol	De Cordt *et al.* (1992)
		Bacillus amyloliquefaciens α-amylase + 83wt%glycerol	701kJ/mol	De Cordt (1994)
		Bacillus amyloliquefaciens α-amylase + 49wt%glycerol +31wt%sucrose	Ea=560kJ/mol[b]	De Cordt (1994)
Chemical				
intrinsic	dispersed	formation of 2,3-dihydro-3,5dihydroxy-6-methyl-(4H)-pyran-4-one	96kJ/mol	Kim & Taub (1993)
extrinsic	dispersed	thiamine breakdown	26°C	Mulley *et al.* (1975a, b)
	Permeable	Maillard's reaction on paper disc	NR	Favetto *et al.*, (1988, 1989)
	Isolated	hydrolysis of disaccharides	18°C	Wen Chin (1977)
		methylmethionine sulfonium breakdown	20-22.8°C	Berry *et al.* (1989)
		acid hydrolysis of sucrose	94.6kJ/mol	Sadeghi & Swartzel (1990)
		destruction of Blue #2 at pH 11.3	58.2kJ/mol	Sadeghi & Swartzel (1990)
		destruction of Blue #2 at pH 9.5	74.5kJ/mol	Sadeghi & Swartzel (1990)
Physical				
extrinsic	isolated	Thermal Memory Cell[b]		Swartzel *et al.* (1991)
		Thermalog S	9.4-9.6°C	Witonsky (1977)

a: in inoculated pack studies, micro-organisms relevant to the type of product and process are added; thus a single z-value cannot be given as this depends on the particular micro-organisms used in a given study.

b: the thermal memory cell is based on the diffusion of at least two different ions, each with their own activation energy, in the insulator layer of a metal-insulator-semiconductor capacitor. Hence, the activation energies that characterize the 'thermal memory cell' depend on the ions used

1: NR: not reported

Table 2.2: State of the art of TTIs (period 1995-2002).

Origin	Application	Principle	Ea (kJ/mol) or z (°C)	Reference
		Enzymic		
Extrinsic	isolated	*Bacillus subtilis* α-amylase at different concentrations in tris HCl buffer pH 8.6 with or without trehalose	6-12°C ($D_{80°C}$ = 9.08 - 33.41 min) ($D_{100°C}$ = 0.14 - 6.16 min) (residual enthalpy reading)	Van Loey *et al.* (1995)
		Bacillus amyloliquefaciens α-amylase (200mg/ml) in tris HCl buffer pH 8.6	7.6°C ($D_{80°C}$ = 112.9 min) (residual enthalpy reading)	Van Loey *et al.* (1996)
		Bacillus subtilis α-amylase at controlled moisture content	9.7°C ($D_{120°C}$ = 14.9 min) (residual enthalpy reading)	Van Loey *et al.* (1996)
		Bacillus amyloliquefaciens α-amylase at controlled moisture content	11.12°C ($D_{121°C}$ = 8.05 min) (residual enthalpy reading)	Haentjens *et al.* (1998)
		Bacillus licheniformis α-amylase at controlled moisture content	10.4°C ($D_{121°C}$ = 19.67 min) (residual enthalpy reading) 8.98°C ($D_{121°C}$ = 16.70 min) (residual activity reading)	Guiavarc'h *et al.* (2002)
	dispersed	Triose Phosphate Isomerase (TPI) in meat	NR[1]	Yih-Chih-Hsu-I (1998)
		Microbiological		
Extrinsic	isolated	*Talaromyces flavus* at different concentrations in polyacrylamide gel in plastic sphere	9.3°C, 10.1°Cor 14.7°C ($D_{80°C}$ between 1.64 min and 7.22 min)	Rönner (1996)
		Bacillus stearothermophilus in beads of alginate+starch+ mushroom puree	NR	Ocio (1997)
		Chemical		
Extrinsic	isolated	Phycoerythrin in borate buffer pH 9. Reading of loss of fluorescence	5.99°C	Orrta-Ramirez *et al.* (1999)
		Nitrophenyl glucoside hydrolysis	21.7°C	Adams & Langley (1998)

1 : NR : not reported.

Table 4.1: D and z values estimates and their associated standard error of regression under isothermal conditions observed with BLA.

	Heat inactivation kinetic parameters	
Temperature (°C)	**D-values (min)**	**Z-value (°C)**
114.9	153.84±6.92	13.32±0.76
120.9	47.39±2.46	
124.7	25.97±1.18	
127.2	18.76±1.43	

Table 4.2: D and z values estimates and their associated standard error of regression under isothermal conditions observed with BSA.

	Heat inactivation kinetic parameters	
Temperature (°C)	**D-values (min)**	**Z-value (°C)**
121.1	54.05±1.85	16.13±0.75
124.9	32.57±1.02	
128.1	19.72±0.67	

Table 4.3: Overview of the kinetic parameters and corresponding absolute average % error for BLA.

DATA	**Regression's type**	**Kinetic parameters $D_{121.1°C}$ (min) Z (°C)**	**Absolute average % error**		
			F_{TTI}/F_{t-T} (z_{TTI})	F_{TTI}/F_{t-T} (z=10°C)	$F_{t-T}(z_{TTI})/Ft-T(z=10°C)$
ISO	Linear	45.77 min 13.32±0.76°C	13.45	13.47	5.99
	Non Linear	52.16±1.56min 14.46±0.57°C	10.94	8.01	6.79
NON-ISO	Non Linear	52.08±0.87°C 9.12±0.67°C	7.91	7.99	1.99

Table 4.4: Influence of storage time on the process-values (F TTI) and their associated standard error derived from TTIs submitted simultaneously to the same heating treatments. (BLA system).

F_{TTI}-storage time	Heating treatments			$D_{121.1°C}$-value* (min)	z-value (°C)
	A: 60 min at 115.8°C	B: 40 min at 121.1°C	C: 15 min at 124.9°C		
F_{TTI}-Day 0*	22.2±1.0	40.0±1.5	28.5±0.8	78.74±3.42	12.85±0.34
F_{TTI}-Day 1*	21.2±0.9	39.7±1.3	24.9±1.5	79.4±2.92	13.51±1.24
F_{TTI}-Day 3*	21.0±1.0	39.9±3.7	23.9±1.0	78.74±8.36	13.86±1.66
F_{TTI}-Day 6*	20.6±1.0	38.5±0.1	26.6±0.6	81.96±0.14	12.82±0.56
% decrease Day 6/Day 0	7.2±6.1	3.8±3.6	6.7±0.5		

* average on 3 values issue from 3 TTIs.

Table annex II.1: Water desorption from a freeze-dried mix stored inside an oven as a function of time and temperature exposure.

N° pan	Initial weight at the output of the dessicator	Storage at 60°C, (*0.01mg) water desorbed/initial weight after			Storage at 102°C, (*0.01mg) water desorbed /initial weight after		
		60 min	120 min	180 min	5 min	10 min	15 min
1	0.23940	-13					
2	0.21941	-12	-12				
3	0.22265	-9	-9	-9			
4	0.21911				-6		
5	0.23202				-7	-14	
6	0.22652				-10	-18	-18

Table annex II.2: Water adsorption on a freeze-dried mix stored at room temperature in function of time.

Details about samples			Initial weight at the output of the dessicator	Storage at room atmosphere (% relative humidity =55%, T=22°C)		Adsorbed water in % of initial mix weight
N° pan	Empty pan	mix (mg)	Total Time 0	(*0.01mg) water adsorbed after 15 min	(*0.01mg) water adsorbed after 90 min	
1	0.17585	0.06365	0.23940	+14	+16	+0.24%
2	0.17731	0.04210	0.21941	+5	+9	+0.21%
3	0.17677	0.04578	0.22255	+8	+12	+0.26%
4	0.17634	0.04277	0.21911	+10	+13	+0.30%
5	0.17841	0.05361	0.23202	+11	+12	+0.22%
6	0.17621	0.05031	0.22652	+8	+8	+0.16%

Table annex III.1: Comparison of FTTI and Ft-T when the capsule is or is not coated with silicone and over-sealed. (*calculated with equation 8).

Run	Coated with silicone?	Sealing	FTTI (equation 2)	F expected=Ft-T (equation 2)	Absolute % error*
1	NO	Normally sealed-1	42.28	15.54	172%
		Normally sealed-2	72.78	15.54	368%
		Normally sealed-3	45.71	15.54	194%
		Normally sealed-4	41.22	15.54	165%
		AVERAGE	**50.49**	**15.54**	**225%**
2		Over-sealed (2.70 mm)	36.70	14.85	147%
		Strongly over-sealed (2.65 mm)	26.33	14.85	77%
		AVERAGE	**31.51**	**14.85**	**112%**
	YES	Normally sealed-5	14.19	14.85	4.4%
		Normally sealed-6	16.36	14.85	10.2%
		Normally sealed-7	14.55	14.85	2%
		AVERAGE	**15.03**	**14.85**	**5.5%**

Table annex IV.1: Processing conditions used to generate conduction heating profiles with a finite difference heat transfer model.

Product properties	
shape	Infinite cylinder
Radius R	0.0015m to 0.0615m by 0.0030m
density	1063 kg/m^3 *
heat capacity	3517 J/kg.K *
thermal diffusivity α	1.67 10^{-7} m^2/s *
initial temperature	20°C (homogeneous)
Processing conditions	
surface heat transfer coefficient coefficient	∞
heating medium	110 to 140°C by 2°C
cooling medium	20°C
$^{10°C}F_{121.1°C}$ (center)	3 min and 8min to 60 min by 4 min

Table annex V.1: Performances of the three multi-component TTIs in products of various fh-value.

fh (min)	Z TTI (°C)	Performances*				
		Abs av	Std abs av	Max neg C	Max pos C	Smallest abs C
10.81	13.5-16.5	12.37	11.18	-7.87	43.09	0.089
	8.5-13.5	4.50	3.96	-16.72	6.168	0.009
	8.5-13.5-16.5	2.28	2.13	-10.108	6.428	0.006
20.11	13.5-16.5	9.435	8.19	-7.62	36.12	0.001
	8.5-13.5	3.14	2.61	-13.296	8.088	0.002
	8.5-13.5-16.5	1.77	1.50	-7.74	7.22	0.006
75.16	13.5-16.5	5.09	4.94	-6.70	42.94	0.053
	8.5-13.5	2.91	1.97	-8.82	9.83	0.003
	8.5-13.5-16.5	2.61	2.02	-2.66	8.69	0.008

*:calculated with 240 values of % correction C.

Abs av: average of the absolute values of the 240 calculated % correction C (in %).

Std abs av: standard deviation associated with Abs av (in %).

Max neg C: highest negative % correction C observed.

Max pos C: highest positive % correction C observed.

Smallest abs C: smallest absolute % correction C observed.

Table annex V.2: Performances of three single-component TTIs in products of various fh-value.

fh (min)	Z TTI (°C)	Performances*				
		Abs av	Std abs av	Max neg C	Max pos C	Smallest abs C
10.81	8.5	21.89	14.81	-33.81	60.85	0.198
	13.5	30.22	18.25	-50.38	77.32	0.104
	16.5	45.56	29.54	-65.74	132.76	0.298
20.11	8.5	19.90	15.93	-26.99	64.29	0.000
	13.5	26.21	15.60	-51.91	65.43	0.249
	16.5	37.56	22.58	-67.48	102.89	0.091
75.16	8.5	24.15	20.02	-10.26	81.26	0.000
	13.5	22.69	15.76	-57.78	20.54	0.166
	16.5	32.26	20.73	-73.67	26.11	0.229

*:calculated with 240 values of % correction C.

Abs av: average of the absolute values of the 240 calculated % correction C (in %).

Std abs av: standard deviation associated with Abs av (in %).

Max neg C: highest negative % correction C observed.

Max pos C: highest positive % correction C observed.

Smallest abs C: smallest absolute % correction C observed.

CLAIMS

1. A time temperature integrator comprising an enzyme, said integrator allowing the evaluation of the impact of a thermal process based on the determination of the residual enzyme activity following the exposure of said integrator to temperatures above 80°C, characterised in that the time temperature integrator comprises a closed container enclosing at least one dehydrated enzyme, an inert carrier and a combination of stabilising substances.

2. A time temperature integrator according to claim 1 characterised in that the moist content of the materials enclosed in the container is below 0.2%.

3. A time temperature integrator according to claim 1 characterised in that the moist content of the substances enclosed in the container is below 0.02%.

4. A time temperature integrator according to claims 1 to 3 characterised in that the sealing of the container is designed to limit the entrance of water in order to prevent that the moist content of the materials enclosed in the container raises above 0.2% before or after the exposure of the time temperature integrator to heat.

5. A time temperature integrator according to claim 3 characterised in that the sealing of the container is designed to limit the entrance of water in order to prevent that the moist content of the materials enclosed in the container raises above 0.02% before or after the exposure of the time temperature integrator to heat.

6. A time temperature integrator according to any of the claims 1 to 5 wherein the integrator comprises the enzyme Bacillus licheniformis α-amylase.

7. A time temperature integrator according any of the claims 1 to 5 wherein the integrator comprises the enzyme Bacillus subtilis α-amylase.

8. A time temperature integrator according any of the claims 1 to 7 wherein the inert carrier is glass, metal or silica.

9. A time temperature integrator according any of the claims 1 to 8 wherein the combination of stabilising substance comprises a pH-buffering substance, a salt and a sugar.

10. A time temperature integrator according any of the claims 1 to 8 wherein the stabilising substance comprises TRIS, sodium chloride, calcium chloride, potassium chloride and sucrose.

11. Use of a time temperature integrator according to any of the claims 1 to 8 to evaluate the impact of a thermal process characterised in that temperatures used in the thermal process range between 80 and 160°C.

ABSTRACT

A time temperature integrator comprising an enzyme, said integrator allowing the evaluation of the impact of a thermal process based on the determination of the residual enzyme activity following the exposure of said integrator to temperatures above 80°C, characterised in that the time temperature integrator comprises a closed container enclosing at least one dehydrated enzyme, an inert carrier and a combination of stabilising substances.

LIST OF FIGURES

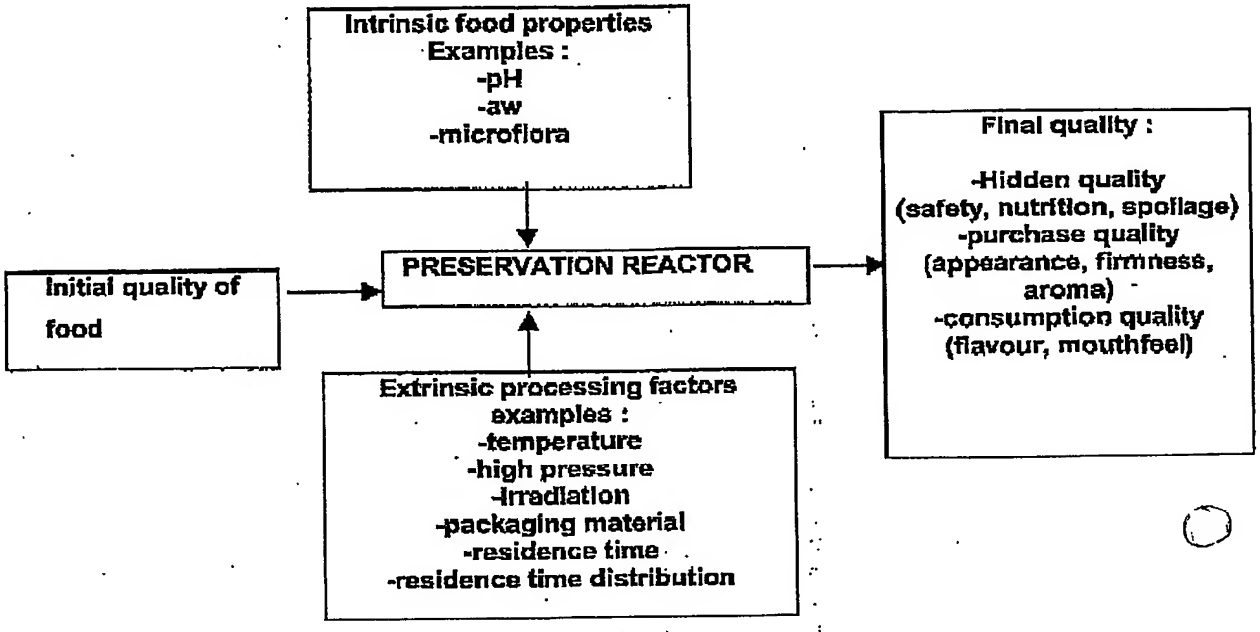


Figure 1.1: The preservation reactor. Example of intrinsic properties and extrinsic factors that can influence the quality of the product are given (Van Loey *et al.*, 1996).

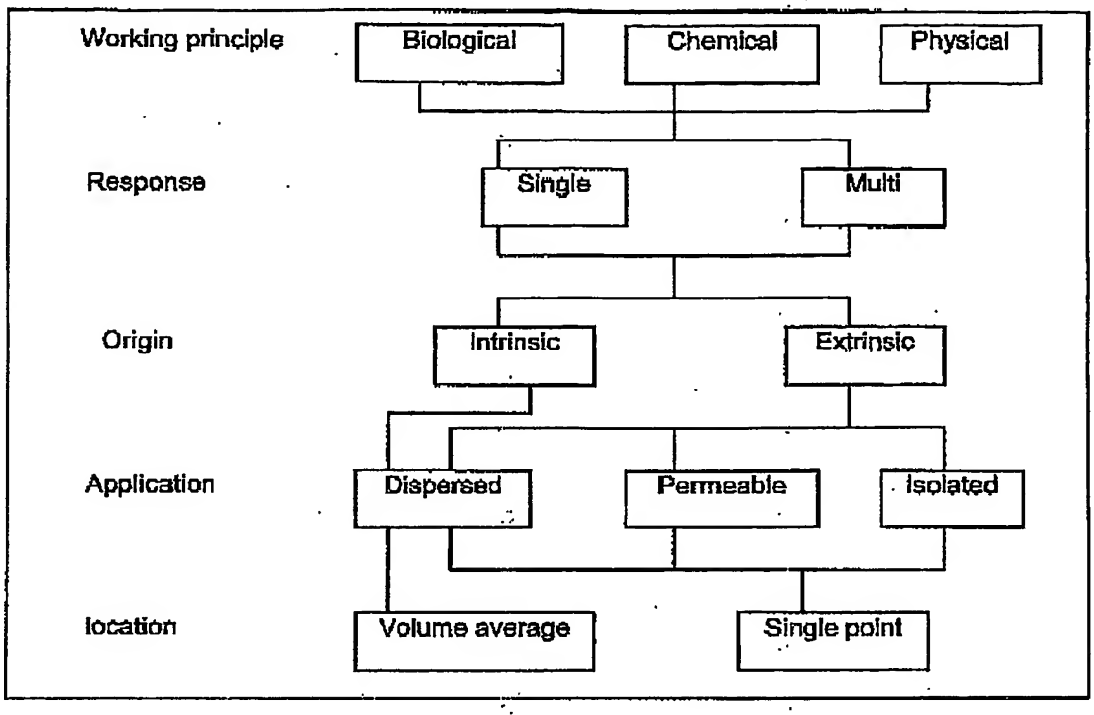


Figure 2.1 General classification of TTIs.

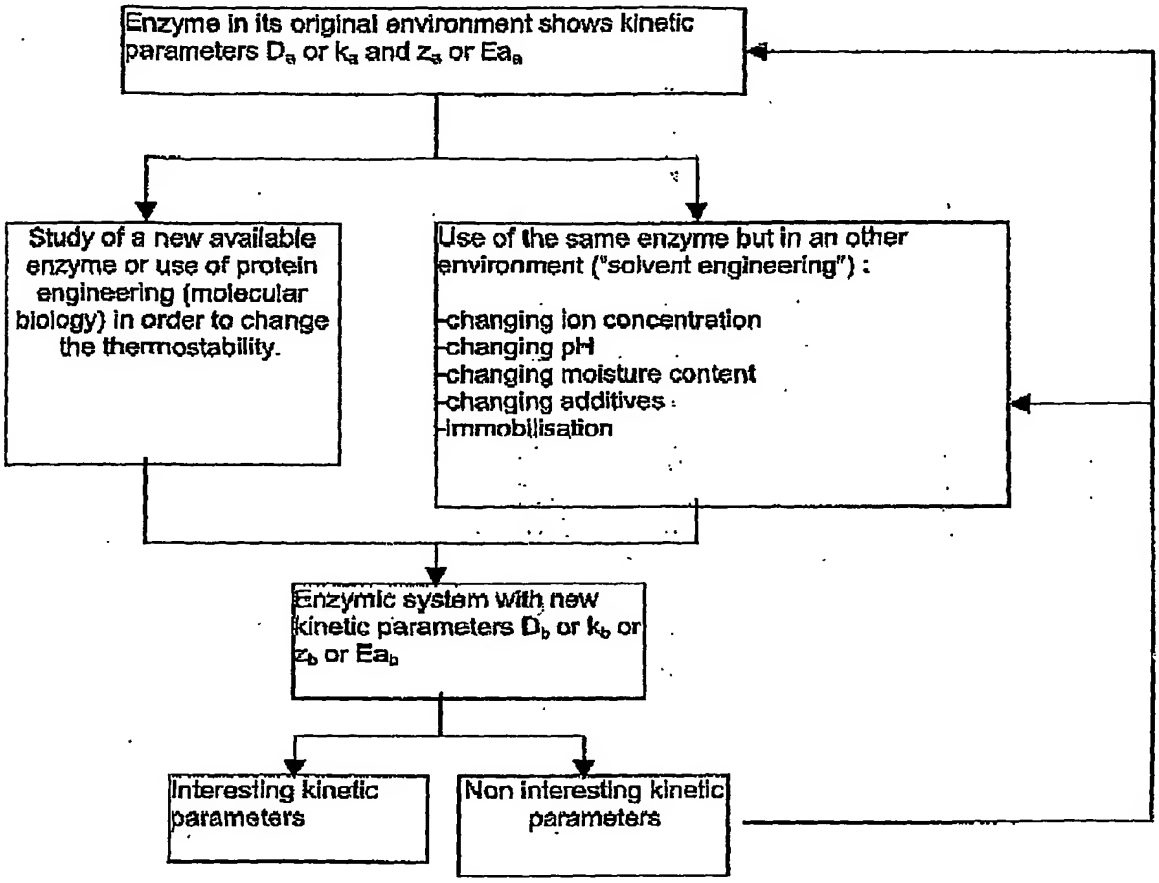


Fig 2.2: Possible approaches to change the kinetic parameters for thermal inactivation of an enzymic system.

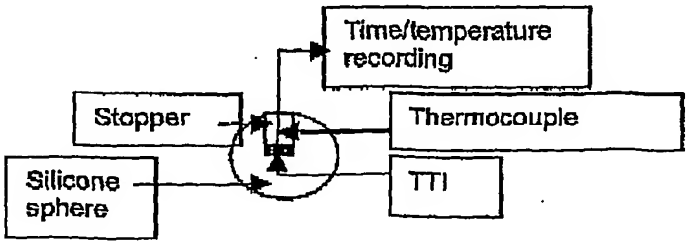


Figure 3.1: System used to validate BLA81 under non-isothermal conditions.

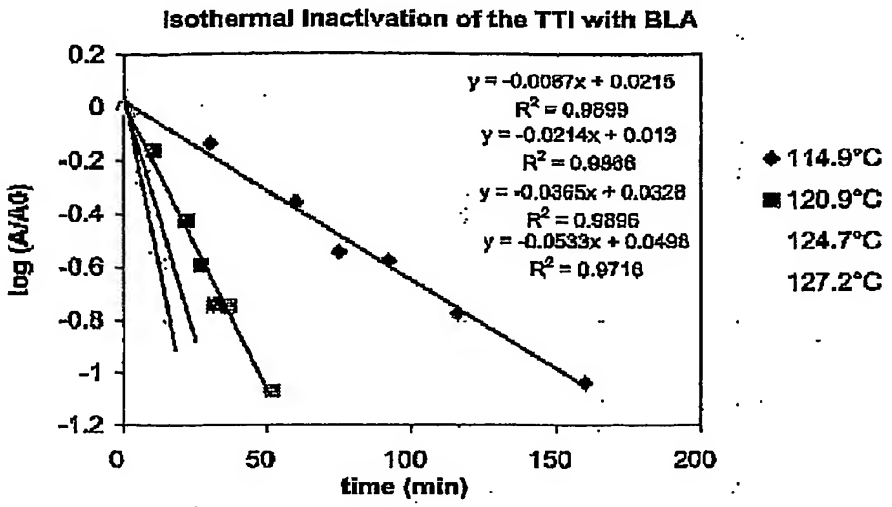


Figure 4.1: First order isothermal inactivation observed with a mix based on BLA

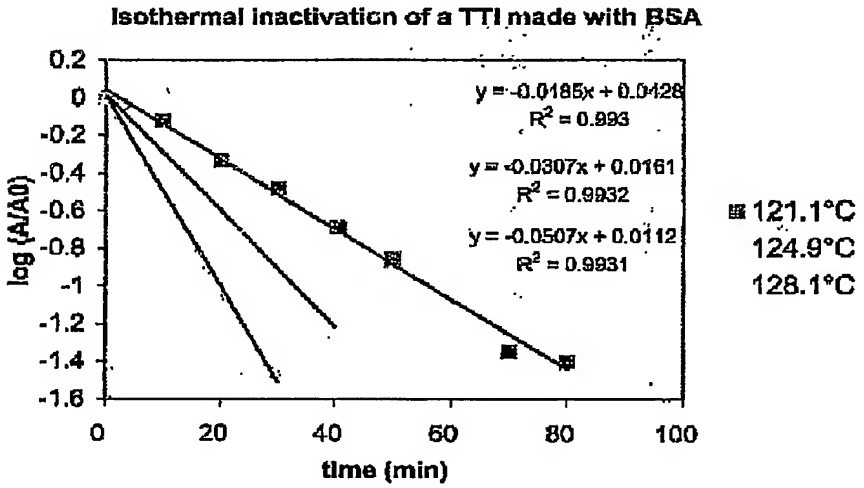


Figure 4.2: First order isothermal inactivation observed with a mix based on BSA

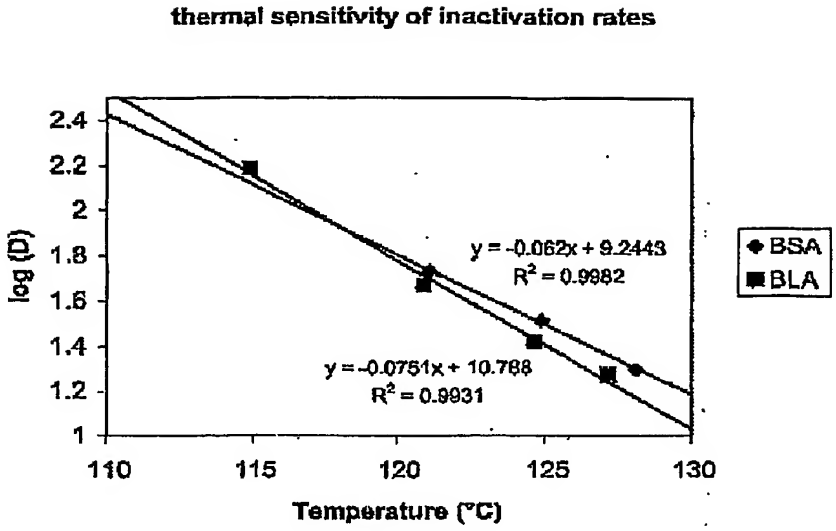


Figure 4.3: Thermal sensitivity of the inactivation rates observed with TTI made with a mix based on BLA or with mix based on BSA.

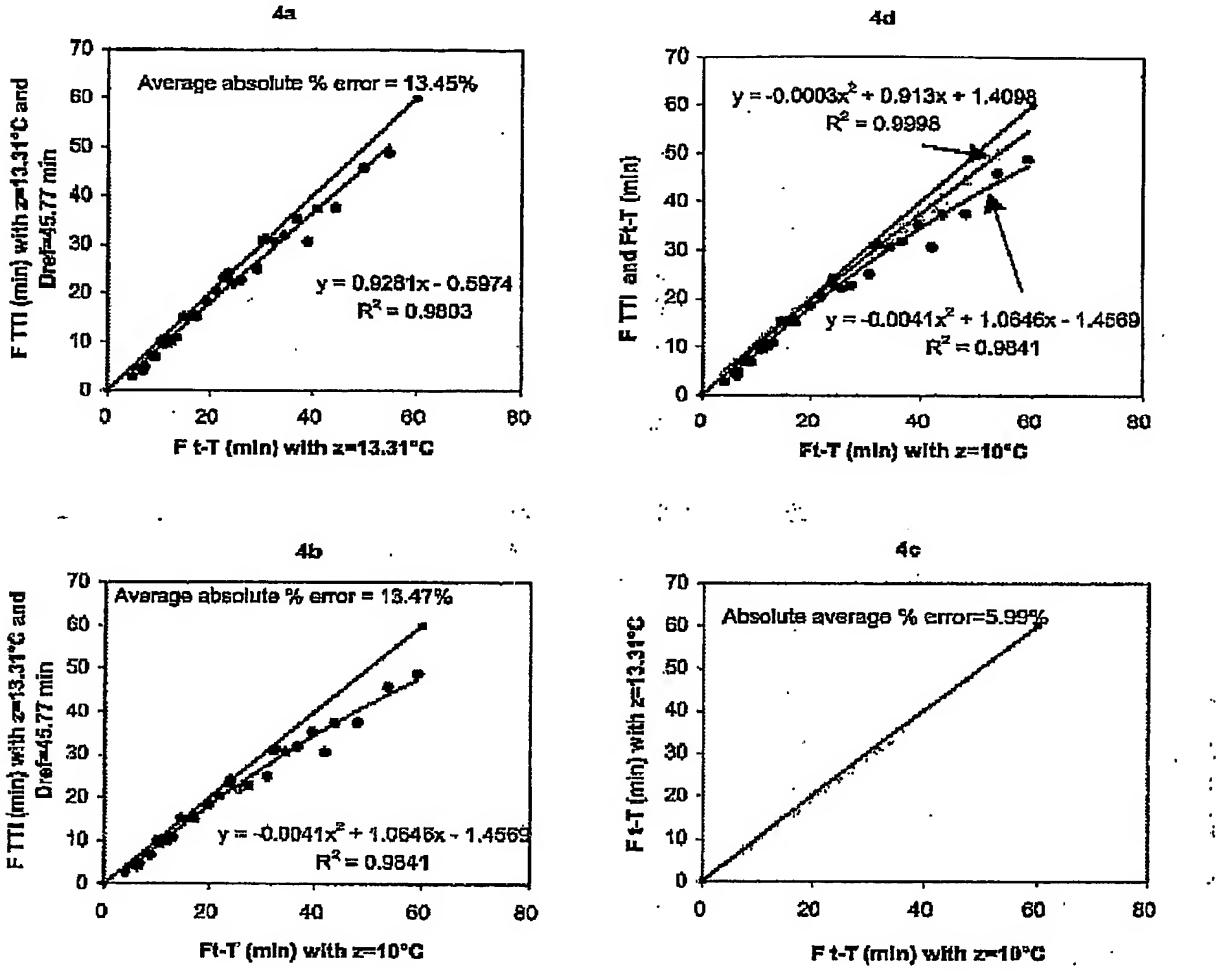


Figure 4.4: observed correlations when using TTI kinetic parameters deduced from a double linear regression method on the isothermal inactivation data (equation 3.2 and 3.3).

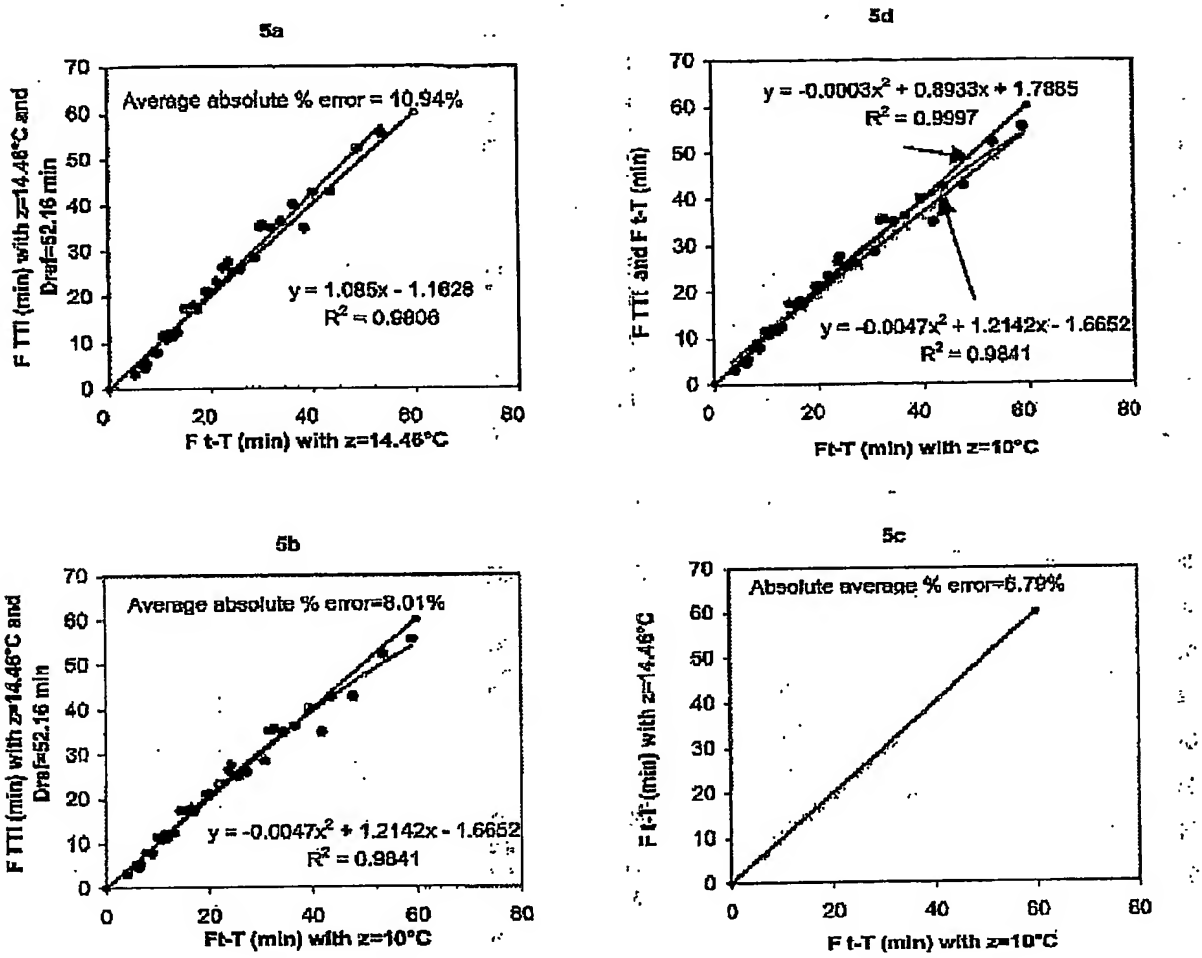


Figure 4.5: Observed correlations when using TTI kinetic parameters deduced from a single step method (non-linear regression : equation 3.4) on the isothermal inactivation data.

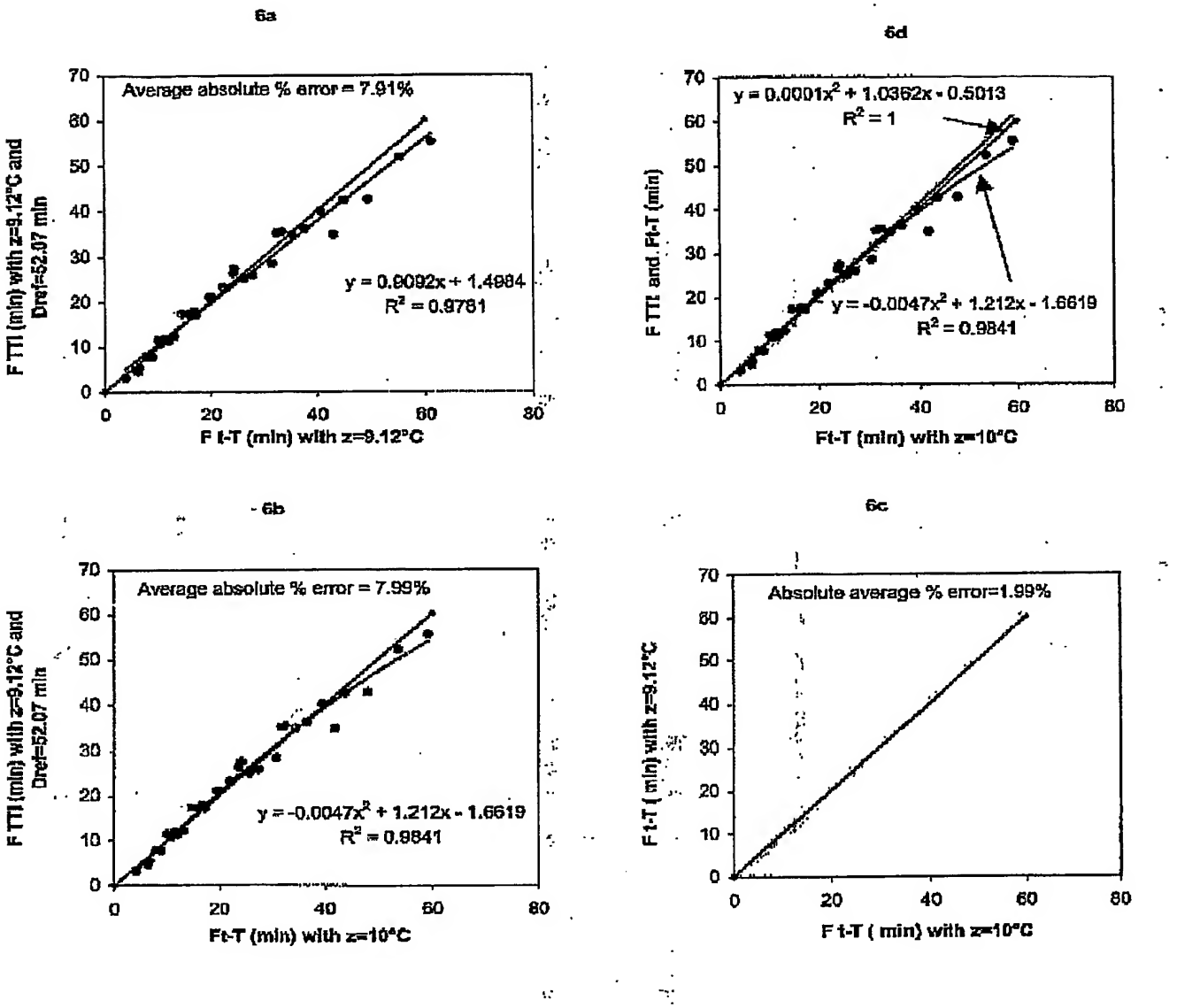


Figure 4.6: Observed correlations when using TTI kinetic parameters deduced from a single step method (non-linear regression : equation 4.1) on the non-isothermal inactivation data.

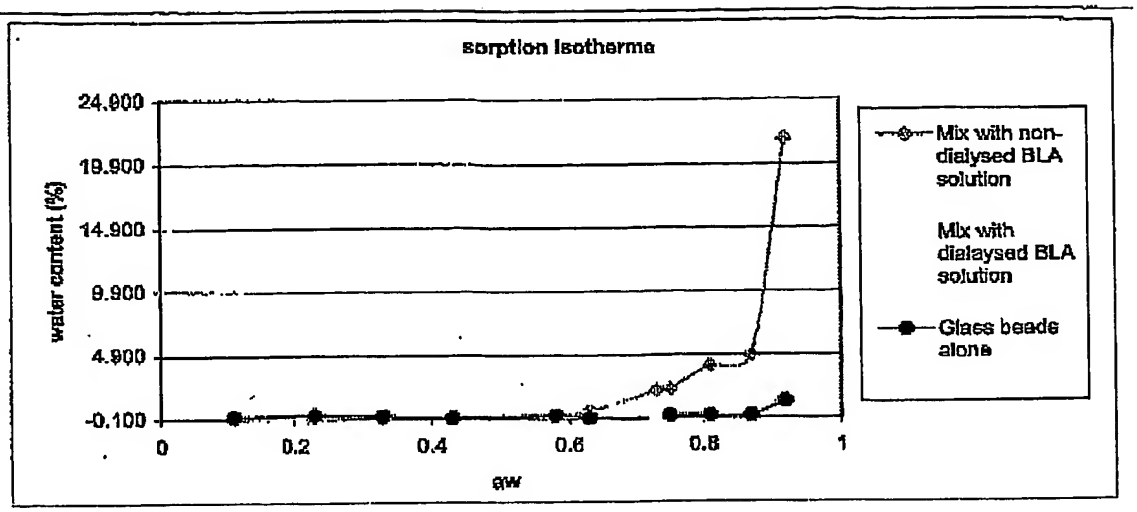


Figure annex II.1: Comparison of sorption isotherms on three different matrices.

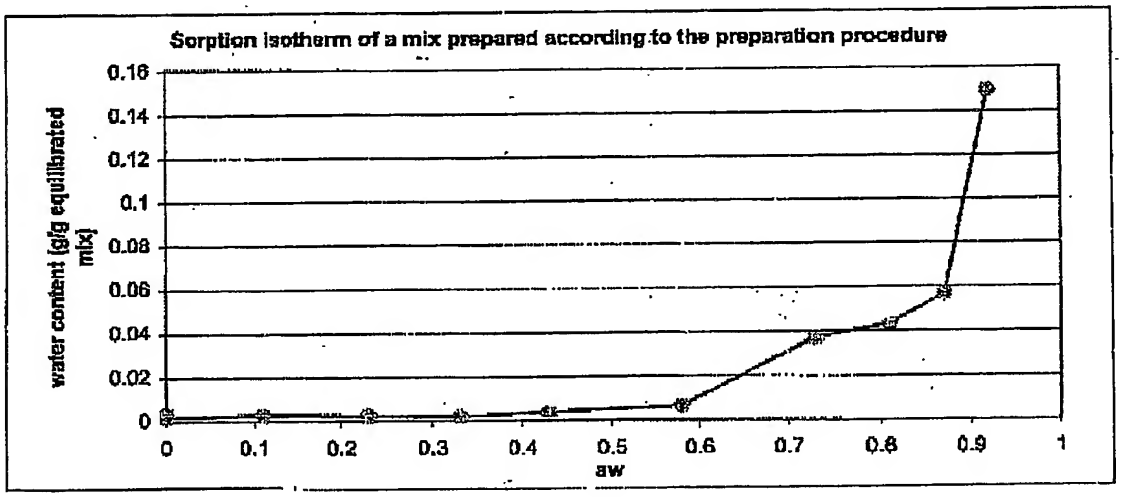


Figure annex II.2: sorption isotherm of a mix prepared according to the TTI preparation procedure with a sample of BLA solution.

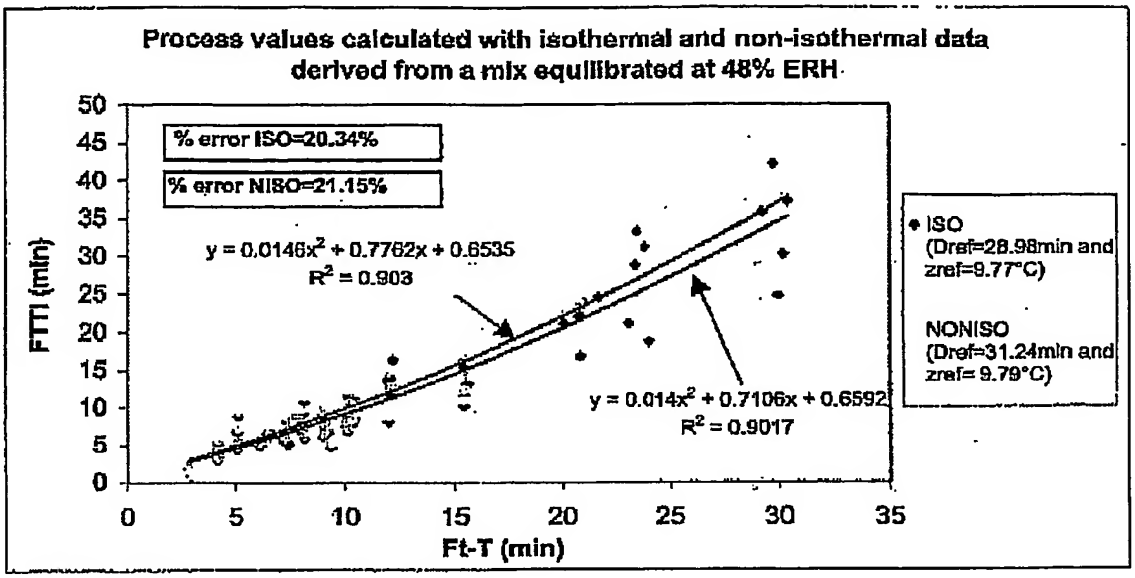


Figure annex II.3: Validation under non-isothermal conditions of a TTI based on a mix prepared with BLA and equilibrated at 48% ERH.

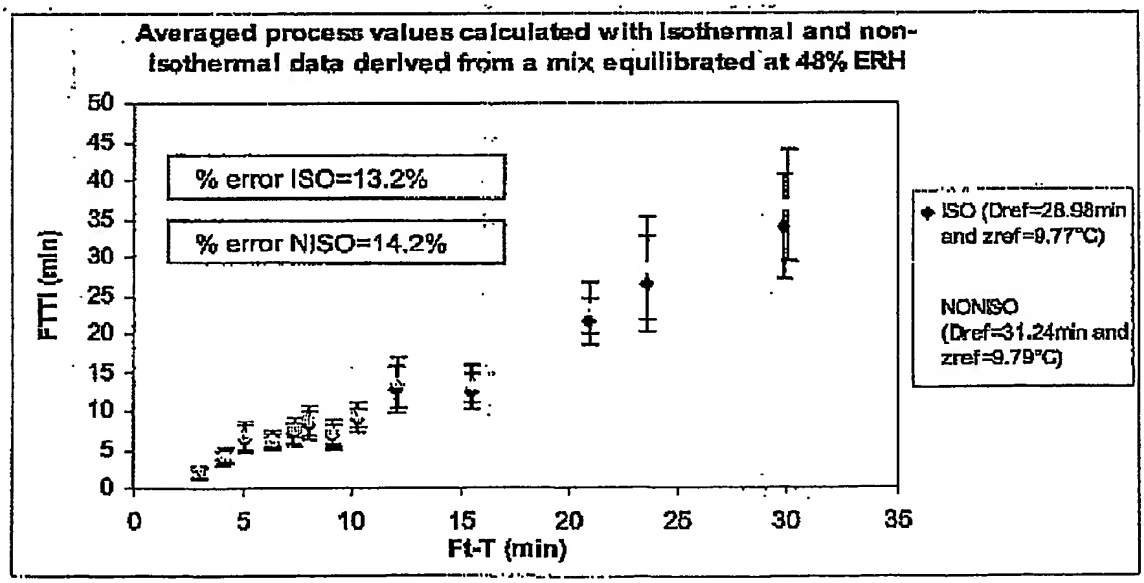


Figure annex.II.4: Validation under non-isothermal conditions of a TTI based on a mix prepared with BLA and equilibrated at 48% ERH. Use of Averaged process values.

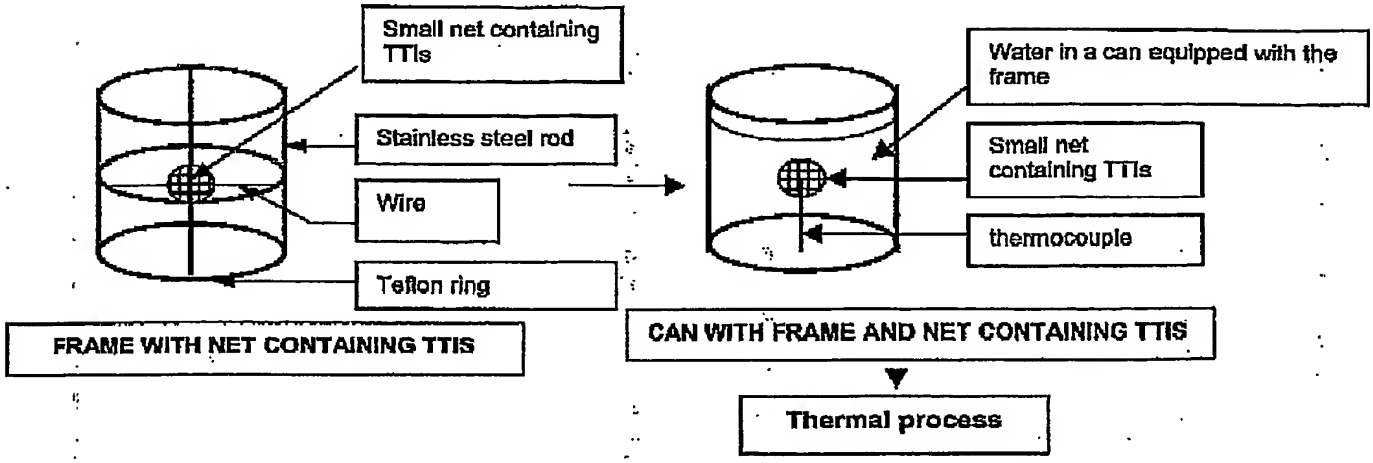


Figure annex III.1: description of a can equipped with a special frame allowing the fixation of a net containing TTIs at the tip of a thermocouple in the center of the can.

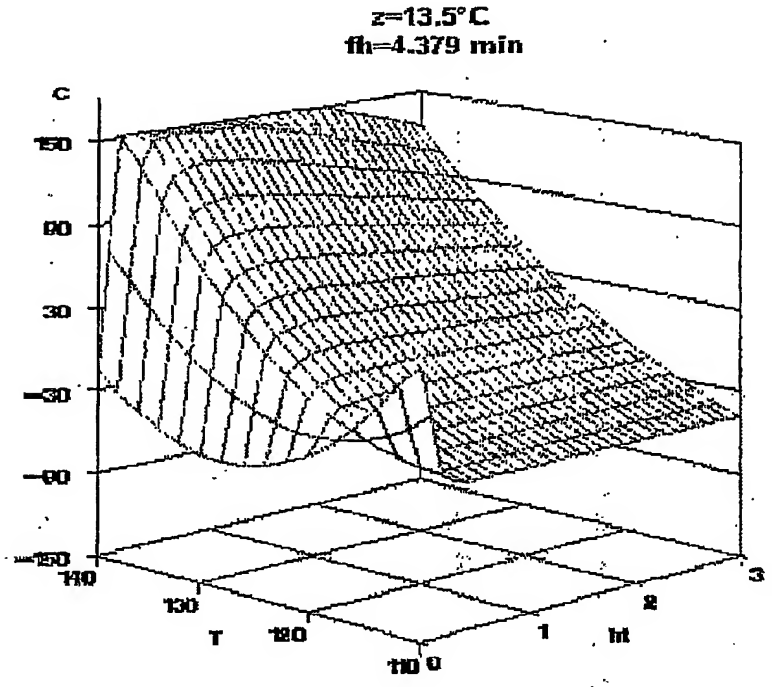


Figure annex IV.1: % correction C to apply to the response of a TTI with a z=13.5°C used in a product with a f_h=4.379 min. ht is the holding time in hours and T the holding temperature in °C.

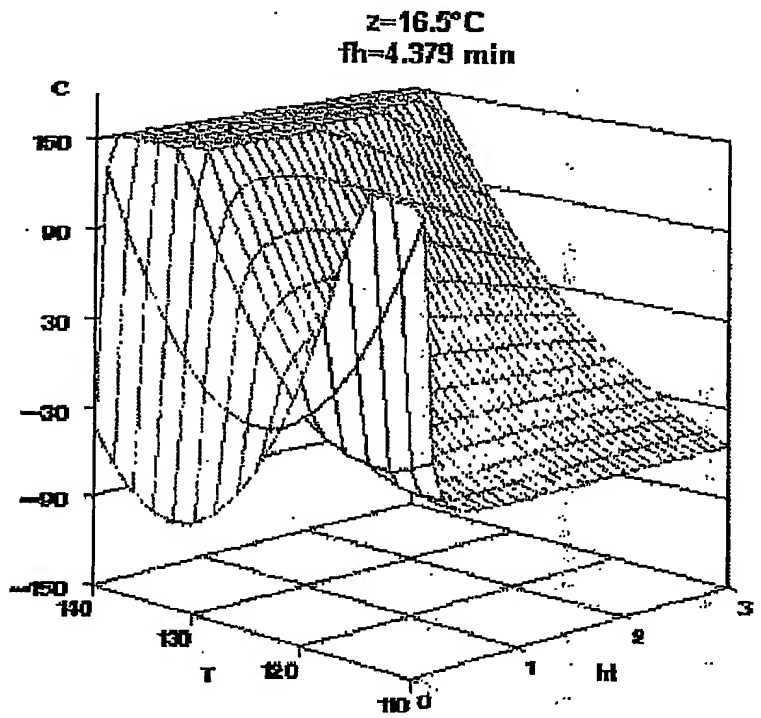


Figure annex IV.2: % correction C to apply to the response of a TTI with a z=16.5°C used in a product with a f_h=4.379 min. ht is the holding time in hours and T the holding temperature in °C.

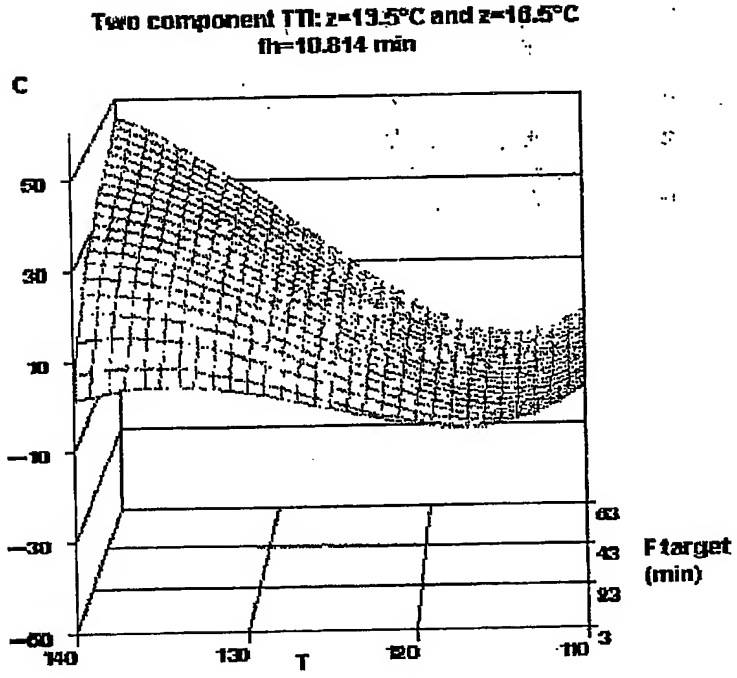


Figure annex V.1: % correction C to apply to the F(multiTTI) deduced from the two component TTI with z=13,5°C and z=16,5°C in a product with fh=10,814 min.

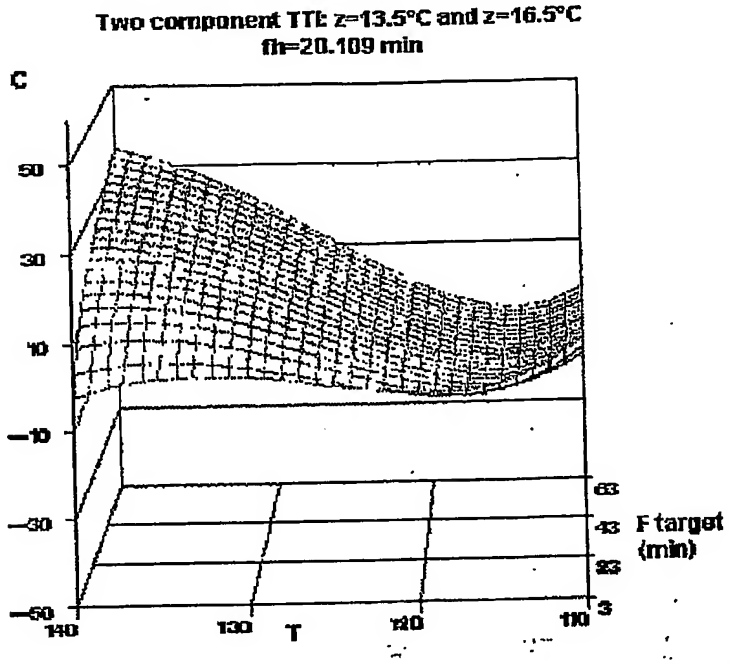


Figure annex V.2: % correction C to apply to the F(multiTTI) deduced from the two component TTI with z=13,5°C and z=16.5°C in a product with fh=20.109 min.

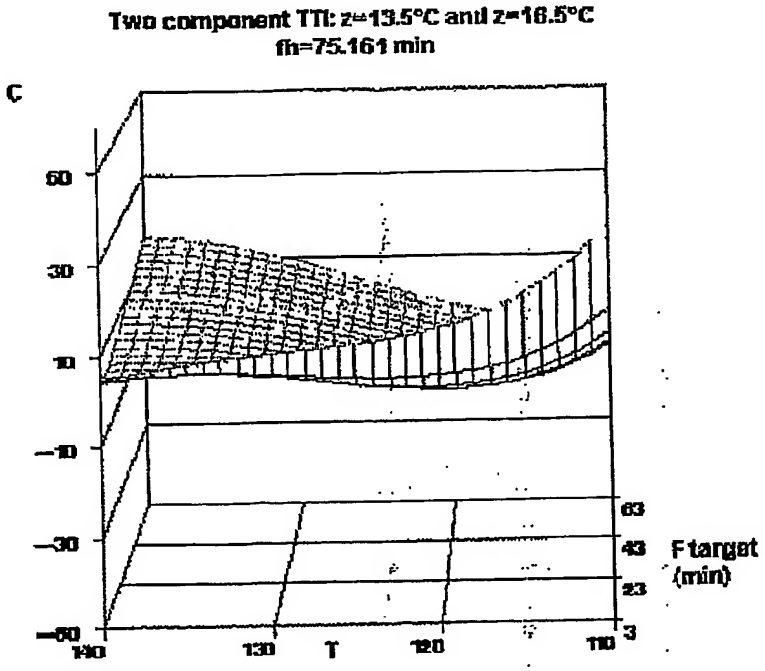


Figure annex V.3: % correction C to apply to the F(multiTTI) deduced from the two component TTI with z=13.5°C and z=16.5°C in a product with fh=75.161 min.

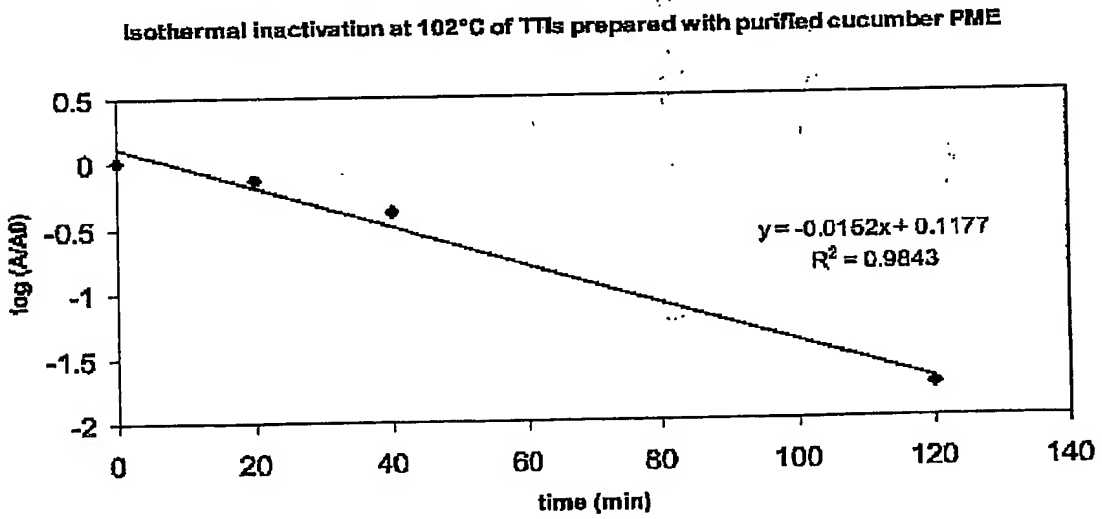


Figure annex VI.1: First order isothermal inactivation observed with a mix based on a purified cucumber PME.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.